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# FACTORS REGULATING HIPPOCAMPAL NEURONAL PROLIFERATION, DIFFERENTIATION AND SURVIVAL

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## DECLARATION

It is authorized the integral reproduction of this thesis, for research purposes.

Jie Lu

**To my mother & father: Defang & Pinzheng**

*To see a World in a grain of sand*
*And a Heaven in a wild flower,*
*Hold Infinity in the palm of your hand*
*And Eternity in an hour*
_____*William Blake*

*I traveled, I saw many things; and I understand more than I can express.*
_____*preacher of Ecclesiastes*

*The only thing that does not change is that everything changes.*
_____*I Ching*

## Abstract

Neural development is controlled by the temporal and spatial coordination of intrinsic and extrinsic factors that may be brain region-specific. In this work, the regulation of postnatal hippocampal neuronal proliferation, differentiation and apoptosis by extrinsic factors including glucocorticoids, neurotransmitters and cytokines was investigated. Hippocampal and cerebellar granule neuronal cultures were used to identify factors which may be responsible for the differential developmental patterns displayed by granule neurons in cells from these morphologically similar brain areas, and the signaling pathways employed were also studied. Other in vitro studies were done to examine the mechanisms underlying glucocorticoid-induced apoptosis in the hippocampus, in particular with regard to the role of glutamatergic transmission.

Results show that transforming growth factor β2 (TGF-β2), supported by the Smad signaling machinery, plays a key role in determining hippocampal cell fate by inhibiting proliferation and, in parallel, inducing neuronal maturation. Brain-derived neurotrophic factor (BDNF), better known for Trk receptor-mediated promotion of neurogenesis and differentiation, was found to exert anti-proliferative and pro-neuronal effects on developing hippocampal neurons by activating a MAP kinase cascade which interacted with the TGF-β signaling pathway.

The glucocorticoid dexamethasone (DEX) was found to directly induce apoptosis in hippocampal cell cultures through the mediation of glucocorticoid receptors (GR); this effect could only be demonstrated if neuroprotective mineralcorticoid receptors (MR) were antagonized. Further, it was shown that mature, rather than immature, granule neurons are targeted by glucocorticoids for apoptosis.

Additional experiments showed that glucocorticoid actions at least partially depend on the prevailing glutamatergic status. The apoptotic actions of DEX are, at least partly, mediated by NMDA and metabotropic glutamate receptors. Low doses of NMDA, acting *via* the *synaptic* NMDA receptor (NMDAR) were shown to efficiently block DEX-induced hippocampal cell death. Evidence was obtained to show that glucocorticoid-induced apoptosis in hippocampal cells is mediated by NMDAR as well as metabotropic receptors. Thus, the final outcome of glucocorticoid treatment on hippocampal cell survival depends on the convergence and integration of transcriptional signals and signals originating at the cell membrane.

In conclusion, these studies have identified some of the factors and signaling pathways contributing to the orchestrated neurodevelopment of hippocampal, as well as cerebellar, neurons.

**Resumo**

O desenvolvimento do sistema nervoso é o corolário de uma coordenação temporo-espacial de vários factores intrínsecos e extrínsecos, que apresentam, adicionalmente, importantes variações regionais. Neste trabalho dissertação foi avaliada a influência de factores extrínsecos, incluindo os glucocorticóides, neurotransmissores e citoquinas, nos mecanismos reguladores da neurogénese (proliferação, diferenciação e apoptose) pós-natal do hipocampo. Foram utilizadas culturas de neurónios hipocampais e cerebelosos para identificar factores e cascatas de sinalização responsáveis pelos padrões distintos de desenvolvimento das células granulares dessas áreas cerebrais. Foram ainda realizados estudos *in vitro* para analisar os mecanismos subjacentes à apoptose hipocampal induzida por glucocorticóides, em particular aqueles que envolvem a transmissão glutamatérgica.

Os resultados obtidos demonstram que o "transforming growth factor β2" (TGF-β2), e a vias de sinalização Smad, desempenha um papel determinante no destino das células hipocampais porque inibe a sua proliferação e, em paralelo, promove a diferenciação neuronal. Ao invés, o "brain-derived neurotrophic factor" (BDNF), conhecido por promover neurogénese e diferenciação neuronal através de receptores tirosina cínase (Trk-r), revelou um efeito anti-proliferativo e favorecedor da maturação neuronal pela activação da cascata da cínase MAP que, por seu turno, interactua com a via de sinalização do TGF-β.

Demontrou-se ainda que a dexametasona, um glucocorticóide, induzia directamente apoptose em culturas de células hipocampais, numa acção mediada pelos receptores dos glucocorticóides (GR); porém, este efeito era apenas evidente se o efeito neuroprotector dos receptores minerolocorticóides (MR) fosse concomitantemente bloqueado. Ficou também demonstrado que apenas as células granulares diferenciadas eram alvo da apoptose mediada pelos glucocorticóides.

Experiências subsequentes permitiram evidenciar que as acções dos glucocorticóides dependiam, pelos menos parcialmente, da neurotransmissão pelo glutamato. Com efeito, a apoptose induzida pela dexametasona era em parte mediada pelos receptores NMDA e metabotrópicos do glutamato. Curiosamente, doses baixas de glutamato, activando os receptores sinápticos do NMDA, bloqueavam eficazmente esse efeito apoptótico. O balanço final do tratamento com glucocorticóides em células hipocampais em cultura depende, pois, da convergência e integração de sinais dependentes da transcrição e de outros com origem na membrana celular.

Em conclusão, estes estudos permitiram a identificação de factores e vias de sinalização que contribuem para a complexa orquestração do desenvolvimento de células hipocampais e cerebelosas.

**CONTENTS**

# Chapter 1

## General Introduction

### 1.1. The life cycle of the neuron

The neuron, the basic function unit of nervous system, is composed of a large cell body and is characterised by its axon and dendrites. Axons and dendrites receive and transmit information using chemical or electrical signals.

During early development (e.g. E14 in rodents), neurons are generated as neuronal precursors from the asymmetrical division of stem cells located in subventricular zone of the brain (Brazel *et al.,* 2003; Alvarez-Buylla *et al.,* 2000). These precursors migrate to their final position following tangential or radial pathways. Neuronal production continues during postntal life in several brain regions, including the olfactory bulb, hippocampus, cerebellum, and cortex; neurogenesis in these various regions varies in intensity (highest during early life) and extends over different periods, e.g. throughout life in the olfactory bulb and hippocampus, and for much shorter periods in the cerebellum and cortex.

Despite high rates of neurogenesis during embryogenesis and early postnatal development, only a proportion of newly-generated cells will survive; most (70% of cortical cells in E14 mouse, Blaschke 1996) die through programmed cell death (apoptosis) due to inadequate neurotrophic support. The surviving cells mature and establish complex neurite extensions and synapses. While most neurons survive for an extended period of the lifespan, granule cells in the olfactory bulb and dentate gyrus of the hippocampus, continually die and undergo renewal from progenitor cell reservoirs.

### 1.2. Neurodevelopmental windows

During development, neuronal proliferation, migration, differentiation and maturation are regulated by multiple intra (intrinsic) and extra (extrinsic) cellular factors, which change in a temporal and spatial way. The most frequently cited case is that of the different cell fates of neural stem cells of different ages or anatomical origins. The differentiation of neurons, astrocytes and oligodendrocytes in the neocortex peaks sequentially before, during and after birth (Sauvageot and Stiles, 2002). The importance of positioning is illustrated by considering that neocortical pyramidal neurons born in the dorsal ventricular zone migrate radially to establish a six-layer- structure in an inside-out manner (Rakic, 1987; 1988); GABAergic neurons and oligodendrocytes originating in the ventral ventricular zone migrate tangentially to their final destinations (Corbin *et al.*, 2001; Parnavelas, 2000; Tekki-Kessaris *et al.*, 2001); and cerebellar Purkinje cells and hippocampal pyramidal neurons arise earlier than cerebellar and hippocampal granule cells (Smeyne *et al.*, 1995; Sonmez and Herrup,1984; Vogel *et al.*,1989; Mullen *et al.*,1997; Altman, 1990; 1965; Harman, 1997). Also of relevance is the observation of GABA and GABA decarboxylase (GAD) in hippocampal granule cells and GABA in mossy fibers until 22-23 days of age, after which their presence is downregaulated with increasing maturity (Gutierrez *et al.,* 2003). All these temporal and spatial differences are controlled by the interplay of intrinsic and extrinsic regulatory molecules.

#### 1.2.1. Intrinsic regulators

The bHLH (basic helix-loop-helix) transcription factors are a large family with ~125 members encoded in the human genome (Ledent *et al.*, 2002). Several of these factors have been shown to control the proliferation and differentiation of cortical progenitor cells (Ross *et al.*, 2003). The transition from proliferation to initial neurogenesis and terminal neuronal differentiation correspond temporally with the differential expression

levels of bHLH factors, from decreased Hes, Id, to increased neurogenin (Ngn), Mash1 and NeuroD (Ross *et al.*, 2003).

- Hes, Id (bHLH differentiation inhibitors) and Ngn, Mash1 (bHLH proneuronal factors) are expressed in the ventricular zone of the telencephalon and trigger differentaition of progenitors there.
- NeuroD, NeuroD2, Nex (bHLH differentiation genes) are expressed in the cortical plate, where fully differentiated neurons are located (Ross *et al.*, 2003).
- Ngns are expressed in the dorsal telencephalon and stimulate the differentiation of glutamatergic neurons;
- Mash1 is expressed in the ventral telencephalon where GABAergic and cholinergic neurons arise (Wilson and Rubenstein, 2000).
- In addition, bHLH gene products interact with other molecules (Notch1, Prox1) to delineate the sequential stages of dentate gyrus development (Pleasure *et al.*, 2000).

Members of the bHLH family have also shown to induce cell cycle arrest by activating cdk inhibitors (Farah *et al.*, 2000), e.g. through the Cip/Kip cdk inhibitors, $p21^{Cip1}$ and $p27^{Kip1}$, which interact with cyclin D-cdk4/6 and cyclin E-cdk2 (Massague *et al.*, 2000). In this context, it is interesting that $p27^{Kip1}$ and the cell cycle retinoblastoma Rb protein (a cdk substrate) were detected in E14.5 progenitors but not E10.5 progenitors in the rat neural tube (Luo *et al.*, 2002), indicating temporal coordination in the expression of the various cell cycle regulator molecules. Indeed, temporal patterns of expression of neurogenesis-regulating genes can be evolutionarily traced back to *Drosophila* in which competence to acquire early-born fates is restricted to mitotic neuronal precursors; in this species, the *Hunchback* gene causes neuroblasts to gradually lose their competence to generate early-born cell types (Pearson and Doe, 2003).

### 1.2.2. Extrinsic regulators

Heterochronic transplants in the ferret cortex showed that transplanted young progenitor cells can adopt the cell fate of the recipient environment, whereas older progenitor donor cells cannot take up the features of a younger recipient; the latter progenitors continue to express characteristics which correspond to their age (Desai and McConnell, 2000), thus implicating a role for regulatory factors in the extracellular environment.

- The transforming growth factor-β (TGF-β) superfamily which consists of more than 35 members, including TGFβ1 and 2, the bone morphogenetic proteins (BMPs), growth differentiation factors (GDFs), activins, glial cell line-derived neurotrophic factor (GDNF), represents a large group of extracellular growth factors that display wide distribution. The functions of members of this superfamily include cell cycle control and differentiation during neural development (Bottner *et al.*, 2000). For example, TGF-β2 was reported to stimulate (Mahanthappa and Schwarting 1993) or inhibit (Constam *et al.*, 1994) neurogenesis, or both (Kane *et al.*, 1996), as well as to regulate neuronal differentiation (Ishihara *et al.*, 1994; Abe *et al.*, 1996; Cameron *et al.*, 1998). TGF-β2 is expressed in a temporal and spatial pattern during development. TGF-β2 levels peak in cerebellar granule cells at postnatal day 10 while these cells are proliferating and migrating; thereafter, their levels decline and they are only expressed in adult cerebellar Purkinje cells (Constam *et al.*, 1994; Unsicker and Strelau, 2000). TGF-β2 immunoreactivity is seen not in the ventricular zone, but is present in the subventricular zone, the telencephalic cortex and the cerebellar anlage of the E16 rat (Flanders *et al.*, 1991). Together, these observations suggest that TGF-β2 contributes to neuronal differentiation.
- In contrast to TGF, the BMP signals block neural maturation and promote an epidermal fate; consistently, BMP mRNA is expressed in epidermal, but not neural cells (Wilson and Edlund 2001). Interestingly, the temporal and spatial patterns of expression of BMPs during mouse embryonic development correspond with its role as a proliferation inhibitor and stimulator of apoptosis in the dorsomedial telencephalon (Furuta *et al.*, 1997). BMP exert both positive and negative effects on

neurogenesis, depending on ligand identity, ligand concentration and the lineage of the responding cell (Furuta *et al.*, 1997; Shou *et al.*, 2000; Zhu, *et al.*, 1999; Mabie *et al.*, 1999; Angley *et al.*, 2003). Expression of both BMP2 and its receptors is highest during embryonic development, with a sharp decline during postnatal life (Mehler *et al.*, 1997).

- Expression of GDNF and its receptors (Widenfalk 1997; Koo 2001) show distinct spatial and temporal patterns. Pinna Serra (2002) demonstrated that GDNF-like immunoreactivity in the neonatal human hippocampus is much higher than in the adult hippocampus. The GDNF receptor C-ret is highly expressed in the dorsal pyramidal cell layer of the hippocampus at P4-P7, declining markedly thereafter. The GDNF receptor GFR-α2 shows a similar expression profile in the cerebellar granule cell layer (Burazin, 1999). GDNF, plays a crucial role in the differentiation, proliferation and survival of neurons, by acting through GFR and RET receptors (Airaksinen and Saarma, 2002). GDNF mRNA and protein levels in the hippocampus increase from early embryonic age to birth, reaching a peak during the first postnatal week and declining to prenatal levels in the adult (Ikeda *et al.*,1999; Lenhard and Suter-Crazzolara, 1998). The mRNA for its receptors are expressed at higher levels in adult hippocampal granule cells than in adult cerebellar granule cells (Burazin and Gundlach,1999).
- GDF10 mRNA expression in rodent brain was observed in the cingulate cortex, retrosplenial cortex, CA3 and ventral limb of the dentate gyrus at P6, persisting in the cingulate cortex up to P21; in marked contrast, GDF10 expression continues to be seen in the hippocampal granule and caudal CA3 cells well into adulthood (Soderstrom and Ebendal 1999).
- Members of the neurotrophin family (e.g. brain-derived neurotrophic factor [BDNF] and nerve growth factor [NGF]) are also representative of extrinsic factors whose distribution follows spatial and temporal patterns; the role of these molecules in the control of neuronal survival, proliferation, and differentiation are well documented. Levels of most neurotrophins increase during ontogeny of the brain, peak around birth and decline in adulthood. They usually show region-specific patterns of distribution, although they may overlap in some areas or at particular stages of development (Rocamora *et al.*, 1993; Zhou *et al.*, 1994).

### 1.2.3. Lifetime events

The birth, maturation and survival of neurons are also heavily under the influence of lifetime events. These may include physiological and cellular stressors, social status, agressivity (Mirescu *et al.*, 2004), nutrition, exercise, pharmacological treatments (Dekosky *et al.*, 1982; Yu *et al.*, 2004; Deisseroth *et al*., 2004), etc. The ultimate effects of the above factors will be determined by the variety of extrinsic and intrinsic factors described above as well as other endocrine, paracrine and autocrine factors; some of these are summarized in the following Tables.

**Regulators of Neuronal Birth – some examples***

| Factors | Effects | System |
|---|---|---|
| **FGF family** | +proliferation +/-Neurogenesis | P0 rat HP,CB; adult SVZ |
| | | Dissociated embryonic rat RT,CTX,HP,ST,SPC; adult SVZ,HP |
| | | |
| **EGF family** | | |
| TGF-α | +proliferation +/-Neurogenesis | Adult rat DG |
| EGF | +proliferation +/-Neurogenesis | Dissociated rat ST,HP,OE; adult SVZ |
| GGF | +proliferation | Dissociated NCSC |
| | | |
| **TGFβ family** | | |
| TGFβ2 | -/+proliferation +/-Neurogenesis | Embryonic rat OE, CB; Dissociated CB granule cell precursors |
| TGFβ3 | +proliferation +Neurogenesis | Embryonic rat RT |
| BMP2,4,5,6,7 | -proliferation +-Neurogenesis | Dissociated rat NCSC, SVZ, embryonic SPC explants |

| | | |
|---|---|---|
| GDF11 | -proliferation +Neurogenesis | Rat OB |
| GDNF | +/-proliferation +Neurogenesis | NT2/D1,SH-SY5Y, C6; Dissociated rat photoreceptors, NCSC, mouse progenitors |
| **Neurotrophins** | | |
| NGF | +proliferation +Neurogenesis | PC12, rat chromaffin cell, DRG, chick sympathetic ganglia embryo |
| BDNF | +/-proliferation +Neurogenesis | Dissociated rat HP; SVZ; CB granule cell, |
| NT-3 | +proliferation +Neurogenesis | Rat DRG, NCSC, sympathetic neuron |
| NT-4/5 | +Neurogenesis | CB granule cell |
| IGF-1 | +proliferation +Neurogenesis | CB granule cell precursors, NSC |
| CNTF/LIF | +/-Neurogenesis | Dissociated fetal/adult rat stem cells |

Table continued on next page –

| | | |
|---|---|---|
| PDGF | +proliferation +Neurogenesis | Rat NSC |
| **Hormones** | | |
| Thyroid hormones, retinoic acid | +Neurogenesis | Dissociated rat photoreceptors |
| Glucocorticoids | -proliferation +Neurogenesis | Rat hippocampal cells in vitro; |
| Mineralocorticoids | +proliferation | Hippocampal cells in adrenalectomized rat |
| Estrogens | -proliferation +Neurogenesis | Mouse embryonic stem cells |
| | | |
| **Excitatory amino acids** | | |
| Non-NMDAR agonist (kainite) | -proliferation | E16/E18 rat CTX explants |
| NMDAR agonist (NMDA) | -proliferation | Adult rat DG |
| NMDAR antagonist (MK801) | +proliferation | P2-5 and adult rat DG |
| | | |
| **Inhibitory amino acids** | | |
| GABA | -proliferation | E16/18 dissociated rat CTX, explants |
| | +proliferation | P6-8 rat CB granule cell precursors; E13-14 mouse VZ |
| **Biogenic amines** | | |
| -Monoamines (αMPT, reserpine) | -proliferation | P11 SVZ |
| -serotonin synthesis inhibitor (pCPA) | -proliferation | E8-12 SC and HP |
| Cholinergic agonist (nicotine) | -proliferation | Embryonic or postnatal CTX |
| | | |
| **Neuropeptides** | | |
| Opioids | | |
| Met$^5$-enkephalin | -proliferation | P6 rat CB |
| Opioid R antagonist (naltrexone) | +proliferation | P6 rat CB, DG, SVZ |
| VIP/PACAP | | |
| PACAP | -proliferation | E13.5 rat dissociated CTX |
| VIP | +proliferation | E15.5 rat dissociated SCG |
| VIP antagonist | -proliferation | E6 mouse prosencephalon |
| | | |
| **Tumour suppressors** | | |
| RB | -proliferation | Rat SVZ, HP subgranular progenitors |
| PTEN | -proliferation | Mouse NSC, |
| SHH | +proliferation | Rat adult HP progenitors, CB granule cell precursors; mouse retinal precursors |
| WNT | +/-proliferation, +neurogenesis | Mouse NPCs, SVZ progenitors, midbrain precursors |

* Based on Cameron HA, Hazel TG & McKay RDG (1998) *J Neurobiol* 36: 287-306.

BDNF, brain derived neurotrophic factor; BMP, bone mophogenetic protein; CB, cerebellum; CNTF, ciliary neurotrophic factor; CTX, cortex; DG, dentate gyrus; EGF, epidermal growth factor; ESC, embryonic stem cells; FGF, fibroblast growth factor; GABA, gamma aminobutyric acid; GDF, growth/differentiation factor; GDNF, glia derived neurotrophic factor; HP, hippocampus; IGF-1, insulin like growth factor-1; LIF, leukemia inhibitory factor; NCSC, neural crest stem cells; NGF, nerve growth factor; NMDA, N-methyl D-aspartate; NPC, neural procursor cells; NSC, neural stem cells; NT, neurotrohpin; OE, olfactory epithelium; PACAP, pituitary adenylate cyclase activating peptide; PDGF, platelet derived growth factor; PTEN, phosphatase and tensin homolog deleted on chromosome 10; RT, retina; SC, superior colliculus; SCG, superior cervival ganglion; SHH, sonic hedgehog; SPC, spinal cord; ST, striatum; SVZ, subventricular zone; TGF, transforming growth factor; VIP, vasoactive intestinal peptide. +, stimulation; -, inhibition.

**Regulators of Neuronal Death – some examples**

| Factors | Functions |
|---|---|
| **Death ligands** | |
| Fas | Bind Fas-L, by recruiting FADD, activate caspase-8, and begin caspase cascade to apoptosis |
| TNF | Bind TNF receptors, the activation of death domain lead to apoptosis by JNK or caspase pathway |
| | |
| **TGFβ superfamily** | |
| TGFβ1 | Protect neuron from apoptosis via c-jun-AP1 pathway |
| GDNF | Promote neuronal survival |
| | |
| **Neurotrophins** | |
| NGF | Promote neuronal survival |
| BDNF | Promote neuronal survival |
| NT-3 | Promote neuronal survival |
| NT-4/5 | Promote neuronal survival |
| | |
| **Excitatory amino acids** | |
| NMDA | Activate NMDA-R, induce calcium influx and apoptosis cascades |
| Kainite | Activate GluR5-7, KA1,2, induce sodium influx and cell swelling |
| AMPA | Activate GluR1-4, induce sodium influx and cell swelling |
| | |
| **Hormones** | |
| Glucocorticoids | |
| Corticosterone | Activate MR and GR, low level is neuroprotective |
| Dexamethasone | Activate GR, induce apoptosis in HP granule cells |
| **Tumour suppressors*** | |
| RB | Promote cell cycle arrest, apoptosis |
| PTEN | Block Akt activity of phosphorylating Bad, pro-apoptotic |
| P53 | G1 phase cell cycle arrest, apoptosis |
| P53BP1 | Responding to DNA-double-strand breaks |
| SMC1 | Intra-S-phase checkpoint; chromosome integrity and the prevention of DNA-damage hypersensitivity |
| NBS1 | Responding to DNA-double-strand breaks; S-phase-cell cycle arrest |
| MDM2 | P53 protein regulation |
| FANCD2 | Intra-S-phase checkpoint |
| CHK2 | Cell cycle arrest, apoptosis and signaling to p53 |
| CTIP | Regulates BRVA1 function |
| BRCA1 | Homologous recombination repair |
| ATM | Autophosphorylation and self activation; coordinating the responses to DNA double-strand breaks |
| | |
| **Apoptosis-related proteins** | |
| Bcl2, Bcl-$X_L$ | Inhibit apoptosis by preventing the release of AIF and cytochrome c from mitochondria |
| Bad | Pro-apoptotic, Dephosphorylation of Bad cause the release of cytochrome c from mitochondria |
| Bax, Bak | Pro-apoptotic, promote mitochondria permeability |
| AIF | Apoptosis inducing factor, induce nuclear condensation and large scale DNA fragmentation in a caspase-independent fasion |
| Caspases | Release of cytochrome c from mitochondria and formation of cytochrome c/Apaf-1/caspase-9 complex initiate caspases activation, cleave downstream substrates and lead to DNA ladder. |
| | |
| **Transcription factors** | |
| NFκB | Inhibit apoptosis |
| IκB | Inhibit NFκB |

* Taken from: Baker SJ & McKinnon PJ (2004) *Nature Reviews Cancer* 4: 184-195.

AMPA, Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; BDNF, brain derived neurotrophic factor; GDNF, glia derived neurotrophic factor; NGF, nerve growth factor; NMDA, N-methyl D-aspartate; NT, neurotrohpin; PTEN, phosphatase and tensin homolog deleted on chromosome 10; TGF, transforming growth factor; TNF, tumor necrosis factor.

### 1.3. Structure, neurochemistry and function of the hippocampus

The hippocampal formation comprises the entorhinal cortex, hippocampus proper, dentate gyrus and subicular complex. The hippocampal formation is an arched or banana-shaped structure, symmetrically located besides the inferior horn of lateral ventrical in the posterior of the dorsal lateral part of the telencephalon, with its long axis extending from the septal nuclei rostro-dorsally to the temporal lobe caudoventrally. The hippocampus proper is composed of the polymorphic layer (hilus), the pyramidal cell layers and the molecular layer **(Fig. 1)**.

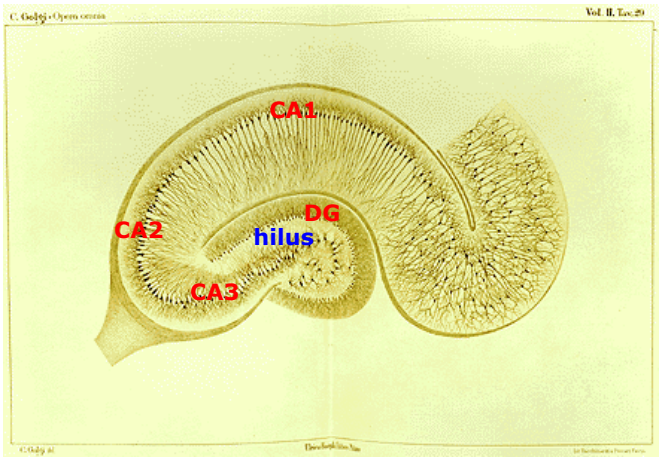


***Figure 1.*** Schematic representation of hippocampus by Golgi, using his silver impregnation stain.

The pyramidal cell layer is divided into the CA1, CA2, CA3 regions, according to differences in cellular architecture: the CA1 region contains small pyramidal neurons while the CA2 and CA3 regions contain larger neurons; the dentate gyrus includes the polymorphic layer and the granular and molecular layers. The granular layer is composed of tightly packed granule neurons, which send mossy fibers to CA3 pyramidal cells. The granule cells receive inputs from the entorhinal cortex via the perforant pathway, and the CA3 neurons project to CA1 neurons via the Shaeffer collateral pathway. Most of the afferent pathways within the hippocampus appear to be glutamatergic, although the hilar-granule connections are mainly GABAergic. Besides perforant pathway, the dentate gyrus also receives cholinergic and GABAergic inputs from the septal nuclei, noradrenergic input from the locus coeruleus, serotonergic input from the raphe nuclei and dopaminergic input from the ventral tegmental area. In addition, the hippocampus expresses several neuropeptides and many of its cells are rich in calcium binding proteins. (Amaral and Witter, 1995; Freund and Buzsaki, 1996)

Together, the inter-connected regions of the hippocampal formation form part of the so-called 'Papez circuit', named after James Papez who proposed a model of interacting brain regions to explain the conscious (cortical-mediated) and peripheral manifestations (hypothalmic- mediated) aspects of emotion (Papez, 1937). This basic plan gave rise to the denomination limbic system, introduced by Paul MacLean who added the following structures: the orbitofrontal and medialfrontal cortices (prefrontal area), the parahippocampal gyrus and important subcortical groupings like the amygdala, the medial thalamic nucleus, the septal area, prosencephalic basal nuclei (the most anterior area of the brain) and a few brainstem formations (MacLean, 1949) (see Fig. 2)

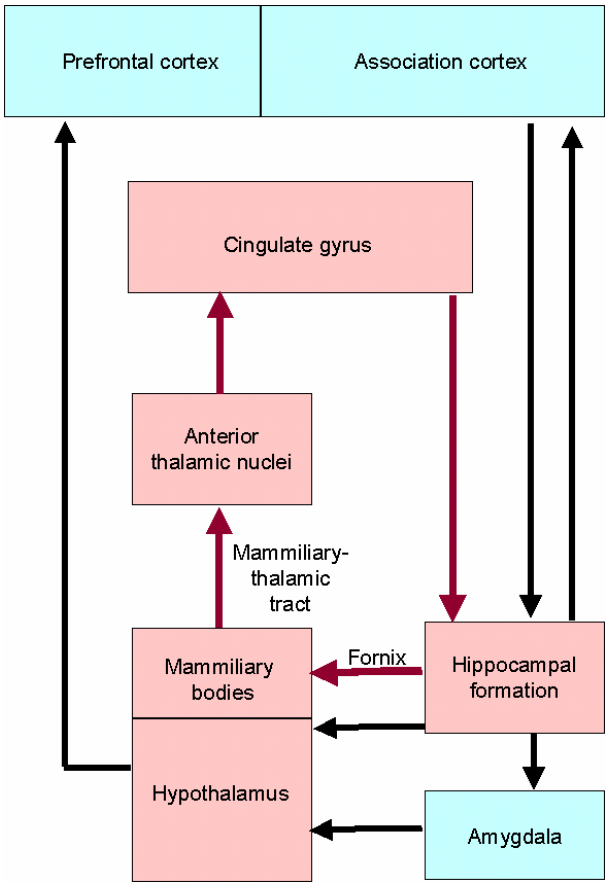


***Figure 2.*** Schematic representation of Papez' circuit, consisiting of the cingulate gyrus, anterior thalamjic nuclei, mammiliary bodies and hypothalamus and the hippocampal formation; some of the other structures added to this circuit by MacLean include the prefrontal and associative cortices and the amygdala.

Recently, Lein, E.S. *et al.* (2004) and Zhao, X. *et al.* (2001) produced what may be called a 'molecular atlas of the hippocampus', based on results obtained by DNA microarray analysis and *in situ* hybridization histochemistry. These authors found certain relationships between the intensity of gene expression and cell morphology. For example, they found that the expression of the *nephroblastoma-overexpressed* gene (*Nov*) is restricted to the CA1 pyramidal layer, the *Purkinje cell protein 4* gene is restricted to the CA2 and dentate gyrus, and *bcl-2 related ovarian killer protein* gene is restricted to the CA3 region. In addition, *desmoplakin* expression was restricted to dentate granule cells, while that of *calretinin* was confined to hilar cells and cells within the subgranular zone of the dentate gyrus, and *Mrg1b* to CA1-CA3 pyramidal cells.

The hippocampus is best known for its role in learning and memory. Evidence for its role in cognition includes experiments showing that damage to the hippocampus leads to severe impairment of declarative (explicit, relational) memory (Squire, 1992). Also, blockade of NMDA receptors and prevention of LTP impair learning of new spatial tasks (Morris, 1989).

The hippocampus also functions as a site of corticosteroid negative feedback, therefore contributing to the neural regulation of the hypothalamic-pituitary-adrenocortical (HPA) axis. Loss of hippocampal neurons or reductions in the levels of glucocorticoid receptor [GR] expression lead to hypercortisolism and DEX resistance, while stimulation of the hippocampus inhibits GC secretion (Sapolsky and Plostsky, 1990). Besides GR, the hippocampus in fact contains another type of corticosteroid receptor, namely, the mineralocorticoid receptor (MR). It is believed that while GR mediate negative feedback signals, MR mediate inhibitory tone on HPA axis activity (Reul *et al.*, 2000).

Lastly, one aspect of hippocampal structure should be mentioned. While the pyramidal cell layers are already formed and post-mitotic at the time of birth, granule cells in the dentate gyrus form just before birth (in the rat), and in all species thus far examined, continue to proliferate throughout life (Kempermann *et al.*, 2004; Arlotta *et al.*, 2003; Seki *et al.*, 2003; Gage *et al.*, 1998). Newly generated granule cells in the adult animal were shown to facilitate synaptic plasticity, thereby contributing to the formation of new memories (Schmidt-Hieber *et al.*, 2004). A number of external enviromental factors, including physical activity, stress and learning influence hippocampal neurogenesis (Prickaerts *et al.*, 2004); stress, for example inhibits proliferation of progenitor cells and has been suggested to be one of the underlying pathophysiological mechanisms of major depression (a condition accompanied by hyperactivity of the HPA axis). Interestingly, anti-depressant drugs stimulate the production of new granule neurons and the latter has been suggested to underpin the therapeutic actions of these drugs (Malberg, 2004; Santarelli *et al.*, 2003).

### 1.4. Neurodevelopmental similarities between the hippocampus and cerebellum

Hippocampal and cerebellar granule cell formation begins towards the end of the embryonic period, with the majority of cells being born during the first postnatal week in rats (Altman *et al.*, 1965, 1972, 1990, Schlessinger *et al.*, 1975). The newly-born cells are formed from precursor cell populations in the secondary germinal matrix (external granular layer, EGL, in the cerebellum; hilus in the hippocampus) which migrate to the proliferative zone without the aid of radial glial guides (Tomasiewicz *et al.*, 1993; Corbin *et al.*, 2001), eventually forming a tightly-packed cellular layer in a phylogenetically ancient cortical structure of the brain (Altman, 1969). While hippocampal granule cell proliferation continues throughout life, albeit at a reduced rate as the organism ages (Kuhn *et al.*, 1996; Cameron & McKay, 2001), cerebellar granule cell production ceases when the EGL disappears during early postnatal life (Altman *et al.*, 1965, 1972, 1990, Schlessinger *et al.*, 1975).

Granule neurons are characterized by highly condensed chromatin and little cytoplasm (Palay & Chan-Palay, 1974, Laatsch & Cowan, 1966, Romon y Cajal, 1911), and express GABA receptors (Tietz *et al.*, 1999, Kim *et al.*, 2000) and glutamate receptors (Wisden *et al.*, 2000, Shigemoto & Mizuno 2000), as well as receptors for other neurotransmitters, neurotrophins and growth factors (see references under the table below). On the other hand, hippocampal and granule cells show some distinct differences; for example, only hippocampal granule cells express the NR2B subunit of the glutamate receptor whereas the NR2C subunit of this receptor are found in their cerebellar counterparts (Wisden *et al.*, 2000). Also, during early development, cancer-related genes are more active in cerebellar granule cells but not in their hippocampal counterparts (Saito *et al.*, 2002). The Table below shows some distinstive and common features of hippocampal and granule neurons.

**Comparison of Cerebellar & Hippocampal Granule Cells**

| | **Cerebellar granule cells** | **Hippocampual granule cells** |
|---|---|---|
| Derivation | Metencephalon[1-4] | Telencephalon[5] |
| Genesis | Begins at the end of embryonic period, reaching a peak during the first postnatal week of life and then stops when EGL disappears [6] | Begins at the end of embryonic period, reaching a peak during the first postnatal week of life; gradually decreases but occurs at low level in adults also [7-9] |
| Migration | Migrate from secondary germinal matrix (EGL) as a precursor population in the absence of radial glial guides [10] | Migrate from secondary germinal matrix (hilar region) as a precursor population in the absence of radial glial guides [11] |
| Morphology | 5-6 μm in diameter, with cytoplasmic indentations in nucleus, highly condensed chromatin, small cytoplasm[12] | 7-10 μm in diameter, nuclear envelope rarely indented, highly condensed chromatin, small cytoplasm [13, 14] |
| Architecture | Tightly packed cellular layer in a phylogenetically ancient cortical structure of the brain[15] | Tightly packed cellular layer in a phylogenetically ancient cortical structure of the brain[15] |
| Glucocorticoids receptors | GR+, MR+ (GR develop later than MR)[16] | GR+, MR+ (GR develop at the same time as MR)[17] |
| Ionotropic glutamate receptor MRNAs[18] | NMDA receptor mRNAs (NR2B switches to NR2C ~ P7)<br>NR1+++<br>NR2A+<br>NR2B-<br>**NR2C+++**<br>NR2D-<br>**KA1-**<br>KA2+++<br>(KA) GluR5-<br>(KA) GluR6+++<br>**(KA) GluR7-**<br>**(AMPA)GluR1-**<br>(AMPA) GluR2+++<br>**(AMPA) GluR3-**<br>(AMPA) GluR4++ | (NR2A and NR2B but not NR2C mRNAs expressed )<br>NR1+++<br>NR2A+++<br>**NR2B+++**<br>NR2C-<br>NR2D-<br>KA1+++<br>KA2+++<br>(KA) GluR5-<br>(KA) GluR6++<br>(KA) GluR7++<br>(AMPA) GluR1+++<br>(AMPA) GluR2+++<br>(AMPA) GluR3+++<br>(AMPA) GluR4+++ |
| Metabotropic glutamate receptor mRNAs[19] | MGluR1+<br>**mGluR2-**<br>**mGluR3-**<br>mGluR4+++<br>**mGluR5-**<br>**mGluR7-** | mGluR1++<br>mGluR2++<br>mGluR3++<br>mGluR4+<br>mGluR5++<br>mGluR7++ |
| Glutamate transporters[20] | EAAT1-<br>EAAT3+ | EAAT1-<br>EAAT3+ |
| Glutaminase[21] | + | + |
| Glycine receptor[22,23,24] | + | + |
| $GABA_A$ receptor mRNA[25,26,27] | **α1++**<br>**α2-**<br>**α4-**<br>**α5-**<br>**α6 +**<br>**β1-+**<br>**β2++**<br>**β3+**<br>γ2+<br>**δ+** | **α1+**<br>**α2++**<br>**α4++**<br>**α5+**<br>**α6 –**<br>**β1++**<br>**β2+**<br>**β3++**<br>γ2+<br>**δ-** |
| 5HT receptors[28,29] | Barely detectable | + |
| Cholinergic receptors (Nicotine)[30,31] | α3+ (low level)<br>**α4+ (low level)**<br>**β2+ (low level)** | + |
| Cholinergic receptors (Muscarine)[32] | **M2+** | **M1+** |
| - Table continued on next page – | | |

| | | |
|---|---|---|
| PKC[33,34] | β,α,ε,**δ** subunits+<br>**(β predominates)** | ε,β,α+<br>**(ε predominates)** |
| Calbindin[35,36] | - | + |
| Dynorphin[37-40] | - | + |
| Transcription factor RU49[41]<br>**(special marker for granule cell)** | + | + |
| NeuroD (critical for granule cell differentiation)[42,43] | + (expression peaks later than in hippocampus) | + (expression peaks earlier than in cerebellum) |
| Cancer related gene expression[44] | **More active at earlier stages** | No such tendency |
| Interleukin 1 beta[45] | + | + |
| NGF & Receptor gp75[45-47] | + | + |
| BDNF & Receptor TrkB[47-49] | + higher than NGF<br>Stimulate migration in vivo | + higher than NGF |
| NT-3 & Receptor TrkC[48,50-52] | + | + |
| NT-4/5[53,54] | + | **protects against adrenalectomy-induced apoptosis of rat hippocampal granule cells** |
| GDNF & Receptor[55-58] | + | + |
| CNTF & Receptor[59-62] | + | ++ |
| AFGF & Receptor[63,64] | + | + |
| - Table continued on next page – | | |
| BFGF & Receptor[65,67] | + | + |
| SCG10 [growth-associated proteins (nGAPs) [68-70] | | + |
| GAP43 [growth-associated proteins (nGAPs) ][69-71] | + | -(in adult rat)<br>+(in monkey) |
| Neuregulins (NRG) [glial growth factor (GGF), acetylcholine-receptor-inducing activity (ARIA), heregulin (HRG) and neu differentiation factor (NDF)] & ErB receptors | **NRG induced NR2C subunit expression**<br>ErbB2, ErbB3 and ErbB4 +[72-75] | |
| Pituitary adenylate cyclase-activating polypeptide (PACAP) &Receptors[76-78] | **Actively expressed in rat cerebellum during postnatal development, peaks between P4-P20; administration of PACAP to P8 rats increases the number of surviving granule cells in the EGL and IGL of the cerebellum, and stimulates neuronal migration from the EGL to the IGL** | PAC(1)-R expression is maintained in adult |
| Polysialylated form of neural cell adhesion molecule (PSA-NCAM) [79-84] | **Granule cells express E-N-CAM on cell bodies, axons, and leading and trailing processes also during migration but cease to reveal detectable levels of E-N-CAM at the end of migration after reaching their final position in the internal granular layer** | Highly expressed during development and persistent expression in the adult rat |
| Bcl-2 mRNA[85, 86] | + (higher in late prenatal development than in postnatal and adult brain) | + (higher in late prenatal development than in postnatal and adult brain) |
| CREB[87-89] | + | **+ Increased cell proliferation is accompanied by activation of CREB phosphorylation in dentate gyrus granule cells** |
| Nuclear factor-kappaB (NF-kappaB), key regulators of either cell death or survival in neuronal cells [90-93] | **high in granule cells before postnatal day 7 (P7) and declines after P7** | + |
| TGF-beta2 [90,94] | **Transient production by postnatal cerebellar**<br>**4-fold up-regulation in P12 versus P4 cerebella, represses NF-kappaB activity** | + |
| - Table continued on next page | | |

| | | |
|---|---|---|
| Glucocorticoid treatment[95-101] | • bFGF↑<br>• No deficits in rotarod performance with Dex treatment<br>• Neonataes treated with hydrocortisone results in decrease cell proliferation and premature cessation of precursors in EGL<br>• **Chronic corticosterone administration does not change Calbindin-$D_{28k}$ mRNA and protein levels**<br>• Decrease GR,MR | • NGF mRNA ↑, bFGF↑<br>• Deficits in Morris water maze performance with Dex treatment<br>• 2 months rat treated with corticosterone for 3hrs decreased cell proliferation<br>• Chronic corticosterone administration increased Calbindin-$D_{28k}$ mRNA and protein levels<br>• Decrease GR,MR |
| Adrenalectomy[99,102-107] | • P11 ADX enhances cell proliferation and delays disappearance of EGL<br>• P22 ADX decreases glutamine, glutamine synthetase, with taurine, aspartic, glutamic and GABA unchanged<br>• **3 months ADX has no effect on Calbindin-$D_{28k}$ mRNA and protein levels in rats**<br>• Increase GR,MR | • P11 ADX enhances cell proliferation<br>• P22 ADX decreases glutamine, glutamine synthetase, with taurine, aspartic, glutamic and GABA unchanged<br>• 3 months ADX decreases Calbindin-$D_{28k}$ mRNA and protein levels in rats<br>• 2 months ADX increases cell proliferation in rats<br>• 3 months ADX induced granule cell loss<br>• Increase GR,MR |
| Transplantation[108-110] | • Neonate cerebellar granule cells switch calbindin gene on when transplanted into neonate hippocampus<br>• Gestation day 14 (E14) cerebellar primordium transplanted into adult rat cerebellum, expression of tenascin, BDNF and NGF-R found during migration and differentiation of grafted Purkinje and granule cells | Adult rat hippocampal progenitors did not express neuronal markers after being transplanted into the adult cerebellum for 8 weeks |

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### 1.5. Problems addressed in this thesis

The questions addressed here may be placed in three major categories:

- Factors contributing to differential neurodevelopmental patterns

  Cerebellum-hippocampus transplantation experiments have suggested the importance of factors released by the donor and/or host cells in neurogenesis (Yoshimura *et al.*, 2000; Tao *et al.*, 1997; Borghesani *et al.*, 2002) and cell fate decisions (Renfranz *et al.*, 1991; Vicario-Abejón, 1995; Suhonen *et al.*, 1996; Alder *et al.*, 1999). Little is known about the identity of the intrinsic and environmental signals that maintain the equilibrium between neuronal birth, maturation and death. Whereas some factors (e.g. bFGF, PDGF) mainly function as promoters of proliferation, others (e.g. TGFβ, BMP, GDF, GDNF) act as inhibitors of proliferation. Like the neurotrophins (e.g. BDNF, NGF), factors such as IGF-1 and GDNF enhance neuronogenesis, whereas factors such as LIF, BMP and CNTF stimulate astrogenesis. In one part of this thesis, we attempted to identify the factors that may be involved in the differential timing of neurogenesis in the cerebelum *vs.* the hippocampus, focussing on the question of whether each brain region may be characterized by specific anti-proliferative and differentiating factors which come into play within

different windows of time. Earlier studies suggested that autocrine or paracrine secretions may play a role in the proliferation, survival and differentiation of developing granule neurons (Gao *et al.*, 1991; Ueki *et al.*, 2003), with a number of experimental paradigms indicating regulatory roles for the neurotrophin BDNF (Lin *et al.*, 1998; Borghesani *et al.*, 2002) and other cytokines (bFGF: Tao *et al.*, 1997; members of the TGFβ superfamily: Unsicker and Strelau, 2000; Pratt and McPherson, 1997; Alder *et al.*, 1999; Angley *et al.*, 2003) in these events. Mumm *et al.*, (1996) demonstrated autoregulation of neurogenesis and, more recently, Wu *et al.*, (2003) showed that mature neurons in the olfactory epithelium secrete a factor (GDF11) that negatively regulates the proliferation of progenitor cells. These questions were addressed using in vitro systms and are described in *Chapter 2*.

- Mechanisms of glucocorticoid-mediated apoptosis in the hippocampus

Glucocorticoids are hormones secreted from adrenal cortex, part of the HPA axis. Corticosterone (in rodents) or cortisol (in humans), by activating GR and MR, play an important role in development and physiological activity. Both receptors are expressed in hippocampus, and granule cell survival and apoptosis are balanced by the activation of these two receptors: MR activated by low coticosterone levels appear to mediate neuroprotective actions, whereas GR which are activated by high corticosterone levels lead to apoptosis of hippocampal granule cells (Almeida *et al.*, 2000). Previous studies showing that the synthetic GR agonist dexamethasone (DEX) can induce granule cell death (Hassan *et al.*, 1996; Almeida *et al.*, 2000) raised interpretational questions requiring demonstration that DEX can directly target hippocampal neurons for death. *Chapter 3* describes the results of experiments designed to solve this question using primary hippocampal cell cultures. The presented results also confirm the view that, because of their neuroprotective properties, activated MR can counteract the cell death-inducing actions of GR.

- Endocrine (adrenal)-neurotransmitter (EAA) interactions

Glutamate is the dominant neurotransmitter in the brain, being found in most excitatory synapses. Its actions are mediated by ionotropic glutamate receptors (iGluR) and metabotropic glutamate receptors (mGluR). The iGluR are directly associated with ligand-gated ionophores permitting $Ca^{2+}$ influx, whereas the mGluR are G protein-coupled receptors which can activate secondary messengers such as cAMP or diacylglycerol and phosphoinositides (Lipton and Rosenberg, 1994; Pin and Duvoisin, 1995). Both types of receptor can activate downstream pathways that determine cell survival or death. Their dichotomous actions appear to result from differences in the duration and magnitude of action and the subsequent levels of intracellular $Ca^{2+}$; thus, synaptic NMDA receptors (NMDA.R) have been associated with the promotion of cell survival and extrasynaptic NMDA.R with apoptosis (Hardingham *et al.*, 2002; Riccio and Ginty, 2002).

Glucocorticoids and GluR share an intimate relationship: (i) glucocorticoids can increase hippocampal cell vulnerability to glutamate receptor (GluR) activation (Armanini *et al.*, 1990); (ii) GR activation leads to an upregulation of the expression of one type of GluR, the ionotropic NMDA receptor (NMDA.R), which is strongly implicated in neurotoxicity (Weiland *et al.*, 1997); (iii) GR occupation has been associated with an increase in glutamatergic transmission (Moghaddam *et al.*, 1994) and (iv) glutamate can enhance GR activation (Gursoy *et al.*, 2001). In light of these interactions, and given the neurotoxic potential of each, glutamate and glucocorticoids, the many possibilities for mutual potentiation of each other's effects, experiments were conducted to explore the mechanisms through which glucocorticoids can modulate glutamatergic effects on hippocampal cell death and survival. The results of these studies are presented in

*Chapters 4* and *5*; the latter chapter also addresses the question of whether immature or mature neurons are targeted by glucocorticoids and glutamate.

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# Chapter 2

## SMAD pathway mediation of BDNF and TGF-β2 regulation of proliferation and differentiation of hippocampal granule neurons

2.1. Supplementary background information

2.2. Lu J., Sousa N. and Almeida O. F. X. (2004). Smad pathway mediation of BDNF and TGF-β2 regulation of proliferation and differentiation of hippocampal granule neurons. *Development.* (Manuscrit under review)

2.3. Additional data

### 2.1. Supplementary background information

The neurotrophin family, whose main members are NGF, BDNF, NT3, NT4/5, play proliferation-, growth- and survival-promoting functions in the central and peripheral nervous systems. These peptide ligands bind to their corresponding tyrosine kinase (Trk) receptors: NGF to TrkA, BDNF to TrkB, NT3 to TrkC, and NT4/5 to TrkB receptors. In turn, the Trk receptors activate downsdream Ras-Raf-MEK-ERK pathways, ultimately influencing the above-mentioned developmental processes, as well as neuronal differentiation and synapse formation and activity.

The TGF-β superfamily is a large family (> 30 members, including TGF-β2, activin, Nodal, and BMP, GDF) of peptides which exert important actions throughout a neuron's life (proliferation, differentiation and apoptosis). Members of the TGF-β superfamily signal by sequentially binding to two transmembrane protein serine/threonine kinases; ligand binding to type II receptors activates type I receptors. Receptor-regulated SMAD proteins (R-SMAD) serve as substrates of type I receptors which subsequently bind Co-Smad4 and translocate to the nucleus, forming a transcriptionally-active complex after association with DNA-binding partner(s). This last complex binds to promoter elements of target genes whose functions include regulation of the cell cycle, differentiation and cell adhesion, positioning, and movement (Moustakas *et al.*, 2001; Chang *et al.*, 2002; Shi and Massagué, 2003). Cross talk between the TGF-β pathway and other signaling pathways are now known, even if incompletely understood. For example, the extracellular receptor kinase (ERK) can phosphorylate some of the Smads. In addition, TGF-β can signal independently of the Smads by activating mitogen-activated protein kinases (MAPKs) such as ERK1/2, p38 and the c-Jun-N-terminal kinase JNK, by activating upstream kinase activators (Attisano and Wrana, 2002).

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2.2. Lu J., Sousa N. and Almeida O. F. X. (2004). Smad pathway mediation of BDNF and TGF-β2 regulation of proliferation and differentiation of hippocampal granule neurons. *Development.*
(Manuscript under review)

# Smad pathway mediation of BDNF and TGF-β2 regulation of proliferation and differentiation of hippocampal granule neurons

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## Abstract

Hippocampal and cerebellar granule cells share several similarities, but while hippocampal granule cells self-renew throughout life, their cerebellar counterparts become post-mitotic during early post-natal development. Here, we show that locally-acting, tissue-specific factors determine the proliferative potential of these two cell types. Thus, conditioned medium from hippocampal cells ($CM_{Hippocampus}$) stimulates proliferation in cerebellar cultures (measured by bromodeoxyuridine incorporation) and, vice versa, mitosis in hippocampal cells is inhibited by $CM_{Cerebellum}$ in a dose- and time-dependent manner. The anti-proliferative effects of $CM_{Cerebellum}$ were associated with an increase in the expression of the cyclin-dependent kinase inhibitors p21 and p27 as well as markers of neuronal maturity/differentiation. Boiling $CM_{Cerebellum}$ resulted in a loss of anti-proliferative activity, indicating its peptidergic or protein nature. Fractionation of $CM_{Cerebellum}$ using ion exchange chromatography yielded fractions with distinct antiproliferative/differentiating and neuroprotective activities. Preadsorption of $CM_{Cerebellum}$ with antisera against candidate cytokines indicated that TGF-β2 and BDNF contribute to the anti-proliferative and pro-differentiating effects of $CM_{Cerebellum}$, an interpretation strengthened by the reproducibility of $CM_{Cerebellum}$ effects with exogenous TGF-β2 and BDNF. $CM_{Cerebellum}$ was shown to enhance nuclear translocation of Smad 2 (transducer of TGF-β2 signaling) and Co-Smad 4. Transient expression of dominant negative forms of Smad 3 and Smad 4 (acting downstream of TGF-β2 specifically) negated the anti-proliferative/differentiating actions of $CM_{Cerebellum}$. BDNF was shown to activate the MEK/ERK pathway, perhaps subsequently TGF-β2 signaling mechanisms. In conclusion, hippocampal neuronal proliferation and differentiation are regulated through an interplay between the paracrine actions of BDNF and TGF-β2.

## Introduction

Control of cell fate, including the mechanisms governing cell proliferation, differentiation and death, is a central theme in developmental neurobiology. Besides contributing to a better understanding of these biological processes, identification of the instructive and permissive factors and their signaling pathways will provide leads for prevention and treatment (including cell replacement) of neurodegenerative and other diseases of the brain. Cerebellum-hippocampus transplantation experiments have suggested the importance of factors released by the donor and/or host cells in neurogenesis (Yoshimura, 2001; Tao, 1997; Borghesani *et al.*, 2002) and cell fate decisions (Renfranz *et al.*, 1991; Vicario-Abejón, 1995; Suhonen, 1996; Alder *et al.*, 1999). Granule cells of the cerebellum and hippocampal dentate gyrus share several morphological commonalities (Ramon y Cajal, 1911); also, they both display dependencies on, or expression of, a common set of growth factors and signaling pathways (Dreyfus, 1998). On the other hand, cerebellar and hippocampal granule cells show distinct differences in their repertoire of glutamate receptor subtypes (Monyer *et al.*, 1994) and gene expression profiles (Saito *et al.*, 2002), features that most likely reflect their different physiological roles. These similar, but also divergent, properties make these two types of granule neuron interesting models for analyzing the intrinsic factors responsible for the regulation of their proliferation and maturation. Both, dentate and cerebellar granule cells first appear late in embryogenesis, with peak numbers appearing during the first postnatal week (Altman *et al.*, 1972; Altman and Bayer, 1990; Schlessinger *et al.*, 1975). Hippocampal granule cells continue to proliferate throughout life, although the rate of proliferation wanes with age (Altman and Bayer, 1990; Kuhn *et al.*, 1996; Cameron and McKay, 2001). In contrast, the genesis of cerebellar granule cells terminates within the first 2 weeks of life (Altman, 1972). Interestingly, gene profiling studies revealed that genes involved in oncogenesis and ribosomal protein synthesis are most strongly expressed at the peak of cerebellar granule cell production (Saito *et al.*, 2002); of the five gene clusters analyzed in that study, none showed any particular temporal pattern of expression in the dentate gyrus.

To examine the hypothesis that tissue-specific factors may serve as 'start' and 'stop' paracrine controls of proliferation in different brain areas, we here measured neurogenesis by the bromodeoxyuridine (BrdU) incorporation in immunochemically characterized cells after exchanging conditioned medium (CM) between rat postnatal day 4 (P4) hippocampal (proliferating) and P7 cerebellar (non-proliferating) granule cell cultures; a similar approach was also used on co-cultures of hippocampal and cerebellar slices derived from differently aged donors. Our studies involving a combination of ion exchange chromatography, immunoneutralization and manipulation of specific signal transduction pathways reveal that TGF-β2 and BDNF secreted from cerebellar granule cells have strong anti-proliferative and neuronal differentiating properties when applied to mitotic dentate granule cells.

## Materials and Methods

### Primary cell cultures and conditioned medium

Hippocampal, cerebellar and cortical primary cell cultures were prepared as previously described (Lu *et al.*, 2003). Briefly, hippocampal (P4) and cerebellar (P7) cells obtained from Wistar rats aged 4 days were digested using the Papain Dissociation System (Worthington Biochemicals); dissociated cells were plated on poly-d-lysine-coated glass coverslips at a density of 400 cells/mm$^2$. Cultures were maintained in Neurobasal A medium/2% B27 Supplement and 1 Mm GlutamaxI and 0.1 mg/ml kanamycin (Invitrogen) in an incubator at 37°C flushed with 5% $CO_2$/95% air and under 90% relative humidity. Half the culture medium was renewed every 3 days. Experiments were started 8-14 days after plating.

Immunocytochemical analysis revealed that the cultures comprised ca. 40% neurons (neuronal markers used: NeuN, TuJ1 and doublecortin), ca. 10% astroglial cells (glial fibrillary acidic protein, GFAP-positive) and ca. 50% progenitor (nestin-positive) cells. Twenty-four hours before experiments, the culture medium was completely replaced with conditioned medium (CM) from either cerebellar ($CM_{Cerebellum}$) or hippocampal ($CM_{Hippocampus}$) cultures containing BrdU (20 Mm). Treated cultures were fixed with 4% *para*formaldehyde (PFA) 24 hours later and processed for the immunocytochemical detection of BrdU.

**Slice culture**

Cerebellar and hippocampal 'interface' slice cultures were prepared from P7 Wistar rats based on a protocol published by Noraberg *et al.* (1999). Briefly, hippocampal and cerebellar slices (400 μm) were placed on Millicell® semiporous membranes in 6-well plates (Millipore). Slices from each brain area were placed adjacent to each other in a single well, and bathed in 50% OPTI-MEM®/Dulbecco's modified Eagle's Medium (DMEM), including 10% fetal bovine serum, 15% horse serum, 1 Mm Glutamax, and 0.1 mg/ml kanamycin in Hank's buffered saline solution; all media and additives were from Invitrogen. Co-cultures were maintained at 37°C (90% humidity) for 16 days, with medium changes every 3 days. Twenty-four hours before fixation (4% PFA), cultures were treated with BrdU (20 μM) and propidium iodide (PI, 20 μM; Molecular Probes).

**HiB5 hippocampal cell line**

Neural precursor SV40 T large antigen-immortalized HiB5 cells (Renfranz *et al.*, 1991) were kindly provided by Dr. Nina Rosenqvist (Lund, Sweden). In all experiments, cells were maintained in DMEM containing 10% fetal calf serum and 1% kanamycin at the permissive temperature (32˚C) and a 5% $CO_2$ environment.

**Immunocytochemistry**

Slice and dispersed cell cultures were fixed in 4% *para*formaldehyde in 0.1 M PBS, permeabilized with 0.3% Triton-X100/PBS and incubated in 3% donkey serum/0.3% Triton for 30 minutes. Specimens were then incubated (1 hour; room temperature) with primary antibodies, all diluted 1:500 in 3% donkey serum/0.3% Triton X-100 in PBS: anti-BrdU (DAKO), anti-Nestin (Chemicon), anti-TuJ1 (Babco), anti-MAP2 (Sigma), anti-doublecortin (Santa Cruz Biotechnology), anti-GFAP (Sigma), anti-αMash1 and anti-αMath1 (kind gifts from Dr. Jane Johnson, Dallas, TX). After washing in PBS, cells and slices were incubated (30 minutes, room temperature) with biotinylated anti-mouse or anti-rabbit secondary antibody (1:500; Sigma). Specimens were then washed thoroughly in PBS and incubated (30 minutes, room temperature) with FITC- or horse radish peroxidase-conjugated Avidin (1:500; Sigma). HRP was developed with diaminobenzidine (0.025% in 0.001% $H_2O_2$ in Tris-buffered saline). In some instances, nuclear staining was achieved using Hoechst 33342 (1:1000 in PBS; 15 minutes; Roche). Specimens were examined using through-light or fluorescence microscopy after mounting in appropriate media. Cells staining positive for BrdU or one of the various neural markers were counted with respect to the total number of cells in 5 randomly-chosen microscopic fields (0.072 $mm^2$; magnification: 400X) across the long axis of each object; an average of 1,000 cells were sampled on each coverslip and the results shown represent values from 6-10 coverslips per treatment.

**Cell death assay**

Cell death was examined in 4% PFA-fixed cells by TUNEL histochemistry or Hoechst 33342 staining. For TUNEL histochemistry, samples were permeabilized (0.1% Triton X-100) and then treated with a peroxidase-blocking solution (1% $H_2O_2$) before being processed as described previously (Almeida *et al.*, 2000). Apoptotic cells were characterized by dark brown nuclear staining; only those nuclei showing evidence of DNA fragmentation without plasma membrane damage were considered to be undergoing apoptosis. Hoechst staining (see above) was used, randomly, to confirm results obtained using TUNEL. The relative number of apoptotic *vs.* Total number of cells were quantified in at least 5 randomly chosen microscopic fields (magnification of 400X).

**Western blotting**

Cells were harvested in lysis buffer, briefly sonicated (ice), and lysates were cleared by centrifugation. Proteins were electrophoretically resolved on 10 or 8% SDS polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were blocked in PBS containing 5% non-fat milk and 0.2% Tween-20, and incubated with specific primary antibodies (anti-MAP2a/b: Sigma, 1:5000; anti-synapsin: Chemicon, 1:400; anti-p21: Pharmingen, 1:500; anti-p27: Santa Cruz, 1:200). Antigens were revealed by enhanced chemoluminescence (Amersham Biosciences) after incubation with appropriate horseradish peroxidase-IgG conjugates (Amersham).

**Concentration and purification of conditioned medium from cerebellar cultures ($CM_{Cerebellum}$)**

A total of 1 L of $CM_{Cerebellum}$ was collected from cultures between 8 and 14 days in vitro (d.i.v.). A 100-fold concentrate, containing peptides with $M_r$ > 6Kda, was prepared using Vivaspin® columns (Vivascience). Concentrated $CM_{Cerebellum}$ was then run through Q-ion exchange columns (Vivapure® 20; Vivascience), and eluted with buffer with a sequential salt gradient. The bio-active fractions were further separated on Affigel blue columns (BioRad). All resulting fractions were analyzed by immunocytochemistry for their ability to influence BrdU incorporation and apoptosis as well as the expression of neuronal markers in hippocampal cultures.

**Heat lability test**

Concentrated (100X) $CM_{Cerebellum}$ was boiled for 15 minutes before addition to hippocampal cultures and measurement of bioactivity (cell proliferation and neuronal markers, as described above).

**Immunoneutralization**

Antibodies against brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) were purchased from Santa Cruz Biotechnology; antibodies against TGF-β2 were from R&D Systems. $CM_{Cerebellum}$ was adsorbed with these antisera for 1 hour (room temperature) before being added to cell cultures at dilutions ranging from 1:10 to 1:10,000 after which the biological activity of the $CM_{Cerebellum}$ was assessed as described before.

**BDNF studies**

Results from the BDNF immunoneutralization experiments were confirmed by adding BDNF (Hbdnf, 10-100 ng/ml; Alomone Laboratories) to hippocampal cultures on 7 d.i.v., and monitoring for BrdU incorporation and MAP2a/b expression after 24 hours.

Further verification of BDNF effects was obtained by transiently transfecting primary hippocampal cultures with a BDNF expression vector (pcDNA3-BDNF-Citron, kindly provided by Dr. Oliver Griesbeck, Martinsried, Germany); Pegfp was used as a control. Transfection was carried out using 1 µg DNA/well and Lipofectamine 2000® (Invitrogen) under serum-free conditions. Twenty-four hours after transfection, media were exchanged between BDNF- and EGFP-transfected cells, and BrdU (20 µM) was added to the cultures. BrdU incorporation was assessed after a further 24 hours.

MAP kinase mediation of the pro-mitotic actions of BDNF was examined by assaying BrdU retention after treating cultures with the MEK1/2 inhibitor UO126 (Cell Signaling; 5 µM). The ability of BDNF (100 ng/ml) to stimulate cytoplasm-to-nucleus translocation of key TGF-β signaling partners was analyzed by transiently transfecting hippocampal cultures with fluorescence-tagged *Smad2* and *Smad4* (see below) and microscopic examination.

**Smad nuclear translocation**

To study nuclear translocation of Smad, primary hippocampal neurons, grown for 7 days in 12-well dishes, were transfected with Pegfp-Smad2 or Pegfp-Smad4 (kindly provided by Dr. Kelly Mayo, Evanston, IL). Transfection was carried out using 1 µg DNA/well and Lipofectamine 2000® (Invitrogen) under serum-free conditions. Transfection efficiency, judged in control transfections with Pegfp, was approximately 5%. Following transfection, cells were returned to standard growing medium or $CM_{Cerebellum}$ for a further 24 hours before staining with Hoechst 33342 and microscopic examination.

**Smad signaling and cell proliferation**

Analysis of Smad signaling in the control of proliferation was studied in primary hippocampal cells after transfection (see above) with 1 µg/well of the following plasmids generously provided by Dr. Joan Massague (New York, NY; Pcs2-FLAG-Smad3 (Smad3 wild type), Pcs2-FLAG-Smad3(3S-A; Smad3 dominant negative), Pcmv5-FLAG-DPC4 (Smad4 wild type), Pcmv5-FLAG-DPC4(1-514; Smad4 dominant negative); Pegfp was used as an internal control. Twenty-four hours after transfection and exposure to either control or $CM_{Cerebellum}$, TGF-β2 (1 ng/ml) or BDNF (100 ng/ml), the number of BrdU-positive cells relative to the total number of (Hoecahst 33342-stained) cells was assessed.

**3TP-Lux reporter assay**

The p3TP-Lux reporter gene, containing a known TGF-β-inducible plasminogen activator inhibitor promoter (Wrana *et al.*, 1994) and kindly provided by Dr. J. Massague, was transfected into HiB5 cells seeded in 24-well plates ($4x10^4$ cells per well), together with wildtype or dominant negative *Smads*. Plasmid (625 ng of total DNA) was introduced using Lipofectamine 2000® into cells maintained in Neurobasal A medium/2% B27 Supplement and 1 Mm GlutamaxI and 1% kanamycin (all from Invitrogen). Twenty-four hours after transfection, cells were treated with $CM_{Cerebellum}$ (10 µl/ml). Cells were harvested 24 h thereafter in 100 µl of 1X lysis buffer (Promega) and centrifuged. The cleared supernatants were assayed for β-gal and luciferase activity. For β-gal detection, 10µl of cellular extract was mixed with 100 µl of β-gal buffer (60 Mm $Na_2HPO_4$, 40 Mm $NaH_2PO_4$, 10 Mm KCl, 1 Mm $MgCl_2$, 2 Mm β-mercaptoethanol) and 20 µl of O-nitrophenyl-β-D-galactopyranoside (Sigma). The reaction was terminated with 50 µl of $Na_2CO_3$ (1M) and luciferase activity was measured by mixing 30 µl of cellular extract with 50 µl of a buffer containing 75 Mm Tris-HCl and 1 Mm $MgCl_2$ (Ph 8). Substrate D-(-) Luciferin (1 Mm) was automatically injected and light emission (410 nm) was measured over 20 s in a luminometer.

### Statistical analysis

All data are depicted as means $\pm$ S.D. and represent the observations from 3-5 independent experiments, with 3-4 replicates for each data point. Data were analyzed for statistical significance using ANOVA and appropriate *post-hoc* tests (Student-Newman-Keuls or Kruskal-Wallis multiple comparison procedures) in which $P \leq 0.05$ was set as the minimum level of significance.

## Results

### Spontaneous neuronal proliferation and apoptosis *(Fig. 1A)*

Hippocampal and cerebellar cultures (8-19 d.i.v.) displayed a dense network of TuJ1-positive neurites (TuJ1 antibody labels neuron-specific tubulin β III). Confirmation that the cerebellar cultures contained post-mitotic neurons was provided by staining for the basic Helix-Loop-Helix (Bhlh) transcription factor Math-1, known to be essential for cerebellar granule cell development (Ben-Arie *et al.*, 1997), and for the neuronal commitment gene Mash-1 (Ross *et al.*, 2003). Hippocampal cultures did not stain for Math-1 but were Mash-1-positive; as in the cerebellum, Mash-1 marks immature neurons in the hippocampus (Pleasure *et al.*, 2000).

Both cerebellar and hippocampal cultures displayed mitosis (BrdU incorporation; blue-black nuclei;) and apoptosis (brown-stained TUNEL-stained cells showing evidence of nuclear fragmentation), shown in the second column of Fig. 1A.

### Cerebellar 'stop' signals *vs.* Hippocampal 'go' signals *(Fig.1B-F)*

To test the hypothesis that cerebellar cells secrete factors that serve as an 'instructive' microenvironment, BrdU incorporation (24 hours) by hippocampal cultures maintained in conditioned medium derived from cerebellar cultures ($CM_{Cerebellum}$) was examined using immunocytochemistry. As shown in Fig. 1B, exposure to $CM_{Cerebellum}$ resulted in a significant reduction in BrdU incorporation ($p < 0.001$). In a reverse experiment, cerebellar cultures treated with $CM_{Hippocampus}$ showed a significant increase in the relative number of cerebellar cells that stained positively for BrdU ($p < 0.01$) (Fig. 1C). Similar results were obtained in studies where hippocampal (4-day old donors) and cerebellar (7-day old donors) slices were used. During the last 24 hours of co-culture (14 days), slices were treated with BrdU (20 μM) and BrdU retention was subsequently monitored by immunocytochemistry. As Fig. 1E shows, cell proliferation in hippocampal slices was significantly reduced in the presence of cerebellar slices as compared to when hippocampal slices were grown alone ($p < 0.01$); in contrast, cell proliferation in cerebellar slices was slightly, but not significantly, increased in the presence of hippocampal tissue.

Besides braking proliferation in hippocampal slices, cerebellar slices also induced cell death in the former, as monitored by the significant retention of propidium iodide (PI) in hippocampal tissue ($P < 0.05$); in contrast, the presence of hippocampal tissue did not influence the number of dying cells within cerebellar slices (Fig. 1F). Representative images of BrdU- and PI-stained co-cultures are shown in Supplementary Figs. IA-D and Figs. 1E-H, respectively.

The above results indicate that soluble factors with mitotic and anti-proliferative properties are secreted into $CM_{Hippocampus}$ and $CM_{Cerebellum}$, respectively. Further, it was shown that the anti-mitotic effects of $CM_{Cerebellum}$ were restricted to hippocampal cells insofar that its addition did not influence proliferation in cortical primary cultures (Fig. 1D). From this point, our studies were focused on the anti-proliferative activity of $CM_{Cerebellum}$ on hippocampal cells.

### Specificity and dose- and time-dependency of $CM_{Cerebellum}$ effects, and physical properties of the

**bioactive moieties present in $CM_{Cerebellum}$ *(Fig.2A, B)***

Incubation of hippocampal cell cultures with varying volumes of 100-fold concentrated $CM_{Cerebellum}$ established that the putative anti-proliferative factors in $CM_{Cerebellum}$ dose-dependently influence BrdU incorporation (Fig. 2A). Further, as shown in Fig. 2B, significant inhibition of BrdU incorporation was observed as early as 3 hours following addition of 10 µl of 100X concentrated $CM_{Cerebellum}$ ($p < 0.001$). Significant inhibitory effects of $CM_{Cerebellum}$ were still observable after 24h ($p \leq 0.05$), but these were absent after 48 and 72 hours. Notably, there was a gradual and significant, time-dependent decline in the proliferative potential of hippocampal cells under basal conditions (fresh control medium *vs.* $CM_{Cerebellum}$). Also, it should be noted that there was no increase in the rate of apoptosis during the first 12 hours after addition of concentrated $CM_{Cerebellum}$ (data not shown).

An insight into the physico-chemical nature of the putative factor(s) contributing to the anti-proliferative activity of $CM_{Cerebellum}$ was gained by assaying the effects of boiled 100-fold concentrated $CM_{Cerebellum}$ on BrdU incorporation by recipient hippocampal cells. As Fig. 2A (last column) shows, the anti-mitotic activity of $CM_{Cerebellum}$ was abolished by boiling for 15 minutes; boiling $CM_{Cerebellum}$ for 5 minutes did not influence biological activity (data not shown). These findings point to the peptidergic/proteinaceous nature of the anti-proliferative moieties present in $CM_{Cerebellum}$.

**$CM_{Cerebellum}$ induces expression of negative regulators of the cell cycle and markers of neuronal differentiation *(Fig.2C-F)***

Western blot analysis showed that treatment of hippocampal cultures with $CM_{Cerebellum}$ induces expression of the cyclin-dependent kinase inhibitors p21 and p27 (Fig. 2C), indicating that the anti-proliferative effects of $CM_{Cerebellum}$ result from its ability to interfere with progression through the cell cycle.

The anti-mitotic effects of $CM_{Cerebellum}$ were found to be associated with enhanced numbers of cells displaying signs of neuronal maturity: hippocampal cultures grown in $CM_{Cerebellum}$ showed a small but significant ($p < 0.05$) decrease in the number of cells immunoreactive for doublecortin (Fig. 2E), the marker of early post-mitotic neuroblasts, and a significant increase ($p < 0.01$) in the relative number of cells that demonstrated MAP2a/b immunoreactivity (Fig. 2D). Increases in MAP2a/b were seen in immunofluorescence and by Western blot analysis. In addition, as also shown in Fig. 2D, hippocampal cultures treated with $CM_{Cerebellum}$ also showed increased expression of synapsin in Western blots. Interestingly, $CM_{Cerebellum}$ had no significant effect on the relative number of glial (GFAP-immunopositive) cells in the hippocampal cultures (Fig. 2F).

The above findings indicate that biological factors present in $CM_{Cerebellum}$ can induce cell cycle arrest within that subpopulation of cells destined to become neurons while, at the same time, accelerating neuronal maturation.

**Presence of multiple factors with differing anti-proliferative, apoptotic and differentiation-inducing properties in $CM_{Cerebellum}$ *(Table 1)***

Working on the premise that more than one factor may account for the anti-mitotic actions of $CM_{Cerebellum}$ on hippocampal cell cultures, concentrated $CM_{Cerebellum}$ was fractionated according to ionic strength after minimizing albumin interference using Affigel Blue chromatography. The 4 fractions that were eluted with NaCl (0.1-1.5 M) showed differing potencies on the proliferative, apoptotic and differentiating potential of hippocampal cells (Table 1). Specifically, fractions eluting at 0.1 M NaCl had significant anti-mitotic ($p < 0.001$) and anti-apoptotic ($p < 0.05$) activities; strong anti-apoptotic activity was observed in eluates containing 1.5 M NaCl ($p < 0.001$), and fractions eluting at 1 M NaCl proved effective at promoting neuronal maturation ($p < 0.05$). These results show that $CM_{Cerebellum}$ contains a cocktail of factors that can differentially influence hippocampal cell fate.

**Identification of anti-proliferative and differentiating factors *(Fig. 3)***

Some of the above experiments indicated that the active factor(s) of interest in $CM_{Cerebellum}$ were heat unstable and of a $M_r$ > 6 kDa. Immunoneutralization was used as a first identification approach; 3 candidate trophic factors, previously implicated in neuronal birth, differentiation and death, namely, NGF, BDNF, and TGF-β2 (Minichilo *et al.*, 1996; Massague *et al.*, 2000; Borghesani *et al.*, 2002; Vaudry *et al.*, 2003) were chosen for analysis of effects on BrdU incorporation and differentiation (MAP2a/b expression). Immunocytochemistry showed the presence of all these peptides in cerebellar and hippocampal cultures (see Supplementary Fig. 2). BrdU incorporation by hippocampal cells was not affected after incubation in anti-NGF-preadsorbed $CM_{Cerebellum}$ (Fig. 3A). Anti-BDNF (1:10) significantly blocked the anti-proliferative effects of crude $CM_{Cerebellum}$ ($p < 0.05$; Fig. 3B), as did anti-TGF-β2 (1:1000 and 1:100; $p < 0.01$; Fig. 3C).

In accordance with our earlier results that showed that $CM_{Cerebellum}$ concomitantly blocks proliferation of hippocampal cells while promoting their maturation (Fig. 2), immunoneutralization against TGF-β2 (antibody concentrations that proved efficient at reversing the anti-mitotic effects) significantly reduced MAP2a/b expression ($p \leq 0.01$; Fig. 3D). In contrast, anti-NGF (which did not influence proliferation) and anti-BDNF did not alter the relative number of MAP2a/b-immunoreactive cells as compared to $CM_{Cerebellum}$ that had not been pre-adsorbed with these antisera (Fig. 3D).

**Replication of $CM_{Cerebellum}$ effects with exogenous BDNF and insights into signaling pathways involved *(Fig. 4)***

Immunoneutralization experiments provide only qualitative information and may be compromised by factors such as antibody affinity and purity. We pursued analysis of the contribution of BDNF by comparing proliferation and maturation in hippocampal cells grown in either $CM_{Cerebellum}$ or normal medium to which BDNF was added. Significant reductions in BrdU incorporation were observed after treatment with 50 ng/ml ($p < 0.05$) and 100 ng/ml ($p < 0.001$) (Fig. 4A).

Similarly, as depicted in Fig. 4B, transfection of hippocampal cells with a plasmid expressing BDNF significantly inhibited cell proliferation compared to cells transfected with Pegfp ($p < 0.001$). Also, when medium from Pbdnf-transfected cells (24 hours) was added to Pegfp-transfected cells, proliferation was inhibited (third bar in Fig. 4B; $p < 0.01$).

In addition to inhibiting cell proliferation, exogenous BDNF at doses between 10 and 100 ng/ml were found to promote neuronal maturation; this was seen as a significant increase in the number of MAP2a/b-immunopositive cells after neurotrophin treatment ($p \leq 0.01$; Fig. 4C). In addition, BDNF (100 ng/ml) stimulated neurite extension ('long neurites' defined as neurites with lengths > twice the diameter of the cell body) within 24 hours of application ($p < 0.01$; Fig. 4C).

The above findings indicate that exogenous BDNF can largely replicate the effects of $CM_{Cerebellum}$, suggesting that BDNF might be an 'upstream player' in the manifestation of $CM_{Cerebellum}$ actions. Since BDNF signaling pathways reportedly converge on those triggered by TGF-β2 following the neurotrophin's activation of ERK1/2 (Segal and Greenberg, 1996; Pera *et al.*, 2003), we pharmacologically tested the involvement of these kinases using the MEK1/2 inhibitor UO126. As shown in Fig. 4D, UO126 significantly abrogated the inhibitory effects of $CM_{Cerebellum}$ on BrdU incorporation ($p < 0.01$). These observations, indicating that BDNF-induced effects on proliferation are mediated through MAP kinases are consistent with earlier reports (Marshall, 1995; Du *et al.*, 2003).

Earlier studies suggested that TGF-β2 signaling pathways may mediate some BDNF actions (Sometani *et al.*, 2001). To confirm that BDNF can initiate a cascade leading to activation of TGF-β2, hippocampal cells were

transiently transfected with 3TP-Lux, a reporter gene containing TGF-ß-responsive elements derived from the plasminogen activator inhibitor 1 (Wrana *et al.*, 1994), and subsequently treated with BDNF. As Fig. 4E shows, BDNF at the highest concentration tested (100 ng/ml) significantly stimulated luciferase activity ($p < 0.01$); similar observations were made in Pbdnf-transfected HiB5 cells (data not shown). Further support that other signaling molecules such as TGF-β2 might contribute to the biological effects of BDNF is provided by the results shown in Fig. 4F: exposure of hippocampal cultures to BDNF led to increased nuclear translocation of Smad2 and Smad4, both of which are crucial for transducing the TGF-β2 signal; pretreatment with cyclohexamide was used to exclude BDNF-stimualted de novo synthesis of proteins (e.g. TGF-β2).

**Exogenous TGF-β2 reproduces effects of $CM_{Cerebellum}$ (*Fig.5A and 5B)***

We also analyzed the contribution of TGF-β2 by comparing proliferation and maturation in hippocampal cells grown in either $CM_{Cerebellum}$ or normal medium to which TGF-β2 was added. Significant reductions in BrdU incorporation were observed after treatment with TGF-β2 at 1 ng/ml ($p < 0.01$) and 10 ng/ml ($p < 0.01$) (Fig. 5A). In addition to inhibiting cell proliferation, exogenous TGF-β2 (1 and 10 ng/ml) was found to promote neuronal maturation, as evidenced by a significant increase in the number of MAP2a/b-immunopositive cells ($p \leq 0.05$; Fig. 5B). In addition, TGF-β2 (1 ng/ml) stimulated neurite extension within 24 hours of application ($p < 0.01$; Fig. 5B).

**Nuclear translocation of Smads after $CM_{Cerebellum}$ treatment *(Fig. 5C)***

TGF-β exerts its biological actions through the mediation of SMAD proteins. Smad2 and Smad3 are specific transducers of TGF-β. Smad2,3 dimerize with Co-Smad4, as do Smads specific to other cytokines (e.g. BMP2-linked Smads1,5,8); these complexes translocate to the nucleus where they influence the transcriptional machinery (Attisano and Wrana, 2002). Here, we demonstrate that transfection of primary hippocampal neurons with either Pegfp-Smad2 or Pegfp-Smad4 followed by exposure to $CM_{Cerebellum}$ results in translocation of the EGFP-tagged proteins to the nucleus (Fig. 5C), indicating that $CM_{Cerebellum}$ contains (an) activator(s) of Smad2 and Smad4. While the Smad2 result strengthens the likelihood that TGF-β is one of these factors, it cannot be ruled out that Smad4 translocation resulted after complexing with Smads activated by other members of the TGF superfamily.

**Dominant negative Smad3 and Smad4 abrogate the anti-proliferative and differentiating effects of $CM_{Cerebellum}$ (Fig. 5D and 5E)**

$CM_{Cerebellum}$ failed to inhibit proliferation in primary hippocampal cells expressing dominant negative forms of *Smad3* (Pcs2-FLAG-Smad3-3SA) or *Co-Smad4* (Pcmv-FLAG-DPC4(1-514) (Fig. 5D), again indicating that TGF-β may be (one of) the major anti-proliferative factor(s) in $CM_{Cerebellum}$. Participation of other Smad-linked factors cannot, however, be excluded, since Co-Smad4 complexes with other members of the Smad family, independently of TGF-β; indeed, since the effects of functional inhibition of Smad4 were significantly greater ($p < 0.01$) than those resulting from Smad3 inhibition, activation of other Smad pathways by non-TGF-β ligands is highly plausible.

In contrast to control-transfected cells, cells expressing dominant negative *Smad3* and *Smad4* showed reduced relative numbers of MAP2a/b-positive cells after exposure to $CM_{Cerebellum}$, demonstrating these Smads to be involved in signaling the effects of (a) maturation-promoting factor(s) present in $CM_{Cerebellum}$ (Fig. 5E). As mentioned before, Co-Smad4 dimerizes with Smad targets that are not activated by TGF-β. Since the inhibition

of Smad4 was here found to have significantly stronger effects on neuronal maturation than inhibition of Smad3 ($p < 0.01$), a role for other Smad-coupled factors cannot be excluded.

**Transactivation of 3TP-Lux following $CM_{Cerebellum}$ treatment *(Fig. 5F-5H)***

As already mentioned, 3TP-Lux is a reporter gene that specifically responds to TGF-β. Hippocampus-derived HiB5 cells (shown to dose-dependently respond to the anti-proliferative effects of $CM_{Cerebellum,}$ Fig. 5F) transfected with 3TP-Lux (Fig.5G) or co-transfected with 3TP-Lux and wildtype *Smad3 or Smad4* (Fig. 5H) responded to $CM_{Cerebellum}$ by driving luciferase expression, an effect was not seen when dominant negative forms of the two Smads were transfected (Fig. 5H). It should be noted that expression of the dominant negative plasmids markedly reduced the luciferase response in cells maintained in control (i.e. not $CM_{Cerebellum}$) medium (Figs. 5H); this finding suggests that HiB5 cells normally secrete a factor (putatively TGF-β) that can transactivate 3TP-Lux. Thus, the immortalized HiB5 cell line appears to be a convenient model for future studies.

## Discussion

The pluripotency of neural cell progenitors (McConnell and Kaznowski, 1991; Coskun and Luskin, 2002) implies that their ultimate phenotype can be influenced by environmental factors. Phenotypic respecification has been demonstrated, for example, in studies involving hippocampal granule cell transplants into the cerebellum (Renfranz *et al.*, 1991) and vice versa (Vicario-Abejón *et al.*, 1995). This adaptive capacity, which indicates an interplay between lineage-specific and extrinsic factors, is gradually lost with time as the host environment becomes increasingly differentiated (Suhonen *et al.*, 1996; Alder *et al.*, 1999), indicating the importance of temporal and spatial organization. Another intriguing aspect of neuronal development concerns the determination of optimal neuronal population sizes. It is recognized that rates of apoptosis and neurogenesis from embryonic development through to adulthood occur in a balanced manner. Earlier studies suggested that autocrine or paracrine secretions may play a role in the proliferation, survival and differentiation of developing granule neurons (Gao *et al.*, 1991; Mumm *et al.*, 1996; Ueki *et al.*, 2003; Wu *et al.*, 2003). Although various experimental paradigms have indicated regulatory roles for the neurotrophin BDNF (Lin *et al.*, 1998; Borghesani *et al.*, 2002) and other cytokines such as Bfgf (Tao *et al.*, 1997) and members of the TGF-β superfamily (Unsicker and Strelau, 2000; Pratt and McPherson, 1997; Alder *et al.*, 1999; Angley *et al.*, 2003) in these events, little is known about the identity of the intrinsic and environmental signals that maintain the equilibrium between neuronal birth, maturation and death.

The appearance and differentiation of cerebellar and hippocampal granule neurons overlap only transiently: cerebellar granule cells enter a post-mitotic state at PND 7-14, i.e. when hippocampal granule cell neurogenesis is at its peak before gradually declining with increasing age (Schlessinger *et al.*, 1975; Altman and Bayer, 1990; Cameron and McKay, 2001). A recent DNA microarray analysis revealed that cerebellar and hippocampal granule cells display distinct gene expression profiles even at times when both cell types are undergoing rapid mitosis (Saito *et al.*, 2002).

In an analogous approach to ones used previously in animals (Renfranz *et al.*, 1991; Vicario-Abejón *et al.*, 1995), the different developmental profiles in the hippocampus and cerebellum were exploited in the present study to identify cerebellar granule cell-specific factors which can reduce hippocampal granule cell proliferation and promote their differentiation. Using granule cell cultures from each brain area, and originating from same-aged animals, and immunocytochemical markers of neuronal maturity (MAP2a/b, TuJ1, Mash1 and Math1) and proliferative potential (BrdU incorporation) we first confirmed that, as compared to hippocampal

cultures, the cerebellar cultures were more mature and mainly post-mitotic after 14 d.i.v. Next, cultures of one type were treated with conditioned medium from the other ($CM_{Cerebellum}$ and $CM_{Hippocampus}$). Analysis of BrdU uptake revealed that whereas $CM_{Hippocampus}$ stimulated proliferation in cerebellar cultures, $CM_{Cerebellum}$ treatment inhibited cell proliferation and accelerated neuronal maturation in the hippocampal cultures; the latter events were accompanied by increased expression of two cell cycle arrest-related molecules, p21 and p27. Similar results were obtained with hippocampus-cerebellum slice co-cultures, as well as when a hippocampus-derived cell line (HiB5) was treated with $CM_{Cerebellum}$. The anti-proliferative effects of $CM_{Cerebellum}$ had a rapid onset (first detectable increases in BrdU incorporation being observed after 3 hours) and were dose-dependent. Together, these results indicate that cerebellar and hippocampal cells secrete cell-type specific factors which have distinct influences on neurogenesis and differentiation. The view that cultures from each brain area secrete unique biologically-active substances was further supported by the finding that the incidence of apoptosis in hippocampal slices was increased in the presence of cerebellar slices, but not vice versa. Lastly, target specificity of these effects was reflected by the fact that $CM_{Cerebellum}$ failed to influence proliferation in cortical cultures.

Hints that the active anti-proliferative/pro-differentiating factor(s) in $CM_{Cerebellum}$ were polypeptidergic in nature were provided by observations that boiling resulted in a loss of biological activity. Importantly, biological potency was retained in $CM_{Cerebellum}$ that was subjected to ion exchange chromatography (fractions contained material with $M_r > 6$ kDa), but the anti-mitotic, apoptotic and differentiating activities eluted at different ionic strengths. Immunoneutralization of the various fractions was subsequently used in a first attempt to identify the active moieties. Our choice of candidates for initial neutralization was based on the differential expression of TGF-β2, NGF and BDNF in hippocampal and cerebellar tissues during development (Unsicker *et al.*, 1991; Sakamoto *et al.*, 1998; Dieni and Rees 2002; also see Supplementary Fig. 2). Those studies showed that neutralization of BDNF and TGF-β2 activities reversed the anti-proliferative actions of $CM_{Cerebellum}$, whereas anti-NGF treatment had no effect on this parameter. At the same time, the pro-differentiating effects of $CM_{Cerebellum}$ were found to be reversed by pre-adsorption of $CM_{Cerebellum}$ with anti-TGF-β2, but not with anti-BDNF or –NGF.

Further studies were focused on verifying the roles of TGF-β2 and BDNF in the observed $CM_{Cerebellum}$-induced effects on hippocampal cell development. Immunocytochemistry demonstrated that BDNF is strongly expressed in cerebellar cultures but only weakly in hippocampal cultures (Supplementary Fig. 2). Treatment of hippocampal cultures with exogenous BDNF resulted in an inhibition of BrdU uptake and an increase in neuritic lengths and the number of MAP2a/b neurons. Transfection of cells with a BDNF-expressing plasmid provided similar results on BrdU incorporation. BDNF effects on neuronal differentiation are known to be mediated through TrkB receptors (Klein *et al.*, 1991) and, depending on the strength and duration of the stimulus, BDNF either promotes or inhibits neuronal proliferation by activating the Trk-MAPK-ERK pathway (Marshall, 1995; Du *et al.*, 2003). This prompted us to examine whether ERK signaling is involved in the biological actions of $CM_{Cerebellum}$. Activation of the ERK1/2 pathway was previously shown to block Smad in *Xenopus* embryos (Pera *et al.*, 2003); in contrast to that finding, we here observed UO126, an inhibitor of MEK1/2 to negate the actions of $CM_{Cerebellum}$ on proliferation. While a plausible explanation for these discrepant findings is lacking, the present data is supported by our observation that BDNF treatment can elicit a response from the TGF-β reporter gene, 3TP-Lux, as well as the ability of BDNF to increase the cytoplasm-to-nucleus translocation of Smad2 and Smad4, two key players in TGF-β signaling. At this stage, the exact intracellular mechanisms that underpin this cross-talk between BDNF-TGF-β remain unknown, but it is

pertinent to mention that previous authors also obtained evidence for cross-talk (cf. Lutz *et al.*, 2004) or interdependence/synergism (cf. Unsicker and Strelau, 2000) between these trophic factors.

There are three isoforms of TGF-β, each derived from separate genes: TGF-β1 whose expression is normally restricted to the choroid plexus, and TGF-β2 and TGF-β3 which are expressed in neurons and glia (Unsicker *et al.*, 1991; Pratt and McPherson, 1997). TGF-β1 and TGF-β3 have been implicated in neuroprotection, while neurotrophic functions have been ascribed to TGF-β2 and TGF-β3 (Finch *et al.*, 1993; Böttner *et al.*, 2000; Pratt and McPherson, 1997). The latter include stimulation (Mahanthappa and Schwarting, 1993) or inhibition (Constam *et al.*, 1994) of neurogenesis, or both (Kane *et al.*, 1996), as well as the regulation of neuronal differentiation (Ishihara *et al.*, 1994; Abe *et al.*, 1996; Cameron *et al.*, 1998). TGF-β2, the isoform focused on in this work, is expressed in the external granular (neurogenic) layer and in Purkinje and radial glia of the cerebellum according to a strict temporal pattern and interestingly, appreciable levels of TGF-β2 are not seen in other brain sites of neuronal proliferation (Flanders *et al.*, 1991; Constam *et al.*, 1994; Unsicker and Strelau, 2000). The expression profiles seen in vivo held true in our cultures: TGF-β2 was much stronger in cerebellar vs. Hippocampal cells (Supplementary Fig. 2). These observations, together with those on the distribution of TGFβ receptors (TGFβR; see below) imply that ligand/receptor availability govern the specific time window within which proliferation and differentiation can occur.

Members of the TGF-β superfamily signal by sequentially binding to two TGFβR which are transmembrane protein serine/threonine kinases; in the case of TGF-β, ligand binding to TGFβR-II activates TGFβR-1; both the developing and rat adult hippocampus express TGFβR-II Mrna (Böttner *et al.*, 1996). Receptor-regulated SMAD proteins (R-SMADs) serve as TGFβR-1 substrates which, upon phosphorylation, subsequently bind Co-Smad4 and translocate to the nucleus where they form a transcriptionally active complex after association with DNA-binding partner(s). This last complex binds to promoter elements of target genes whose functions include regulation of the cell cycle, differentiation and cell adhesion, positioning, and movement (Moustakas *et al.*, 2001; Chang *et al.*, 2002; Shi and Massagué, 2003). For example, cell cycle arrest by TGF-β involves suppression of the oncogene Myc, a repressor of the cyclin-dependent kinase inhibitors, p21 and p27 (Seoane *et al.*, 2002; Gartel and Shchors, 2003). Supporting the view that TGF-β2 may be responsible for at least some of the antimitogenic activity of $CM_{Cerebellum}$ we here observed an upregulation of p21 and p27 after $CM_{Cerebellum}$ treatment of proliferating hippocampal neurons.

Additional evidence for a key role of TGF-β2 in the hippocampal cell fate-determining actions of $CM_{Cerebellum}$ was obtained in a series of experiments focused on the TGF-β signal-propagating SMAD proteins. Of the various members of the SMAD system, Smad2 and Smad 3 mediate TGF-β signals, but also those of activin, another member of the TGF-β superfamily. Smad4 is a requisite partner for transcriptional activity of Smads2 and 3, as well as Smads1, 5 and 8 which are substrates of the bone morphogenetic proteins (BMP); the generation of specific downstream responses presumably depends on the formation of particular R-SMAD-Smad4 complexes which then recruit different sequence-specific DNA-binding factors (Massagué and Wotton, 2000). Here, we demonstrated that $CM_{Cerebellum}$ treatment can induce nuclear translocation of EGFP-Smad2 and –Smad4. Consistent with previous results showing that Smad2 translocation does not require Smad4 (Liu *et al.*, 1997), we observed that translocation occurred to a similar extent in cells transfected with the individual plasmids or co-transfected with *EGFP-Smad2* and *GFP-Smad4*. Essential roles for Smad3 and Smad4 were also demonstrated insofar that expression of the dominant negative forms of either of these molecules prevented the transactivation of 3TP-Lux by $CM_{Cerebellum}$ and abrogated the anti-proliferative and pro-differentiating effects of $CM_{Cerebellum}$ in hippocampal cultures.

In summary, we have demonstrated that TGF-β2, acting in a paracrine fashion, plays a key role in determining hippocampal cell fate, namely by inhibiting cell proliferation and promoting neuronal differentiation. TGF-β2 is expressed at moderate levels in the adult hippocampus (Unsicker *et al.*, 1991) but is only weakly detectable by immunocytochemistry in early postnatal hippocampal cells; however, the developing hippocampus appears to have all the signaling machinery required to respond to TGF-β2 of extra-hippocampal origin. Our studies also show that BDNF, better known for Trk receptor-mediated promotion of neurogenesis and differentiation (Klein *et al.*, 1991; Gao *et al.*, 1995; Pencea *et al.*, 2001), can exert anti-proliferative effects on hippocampal granule cell neurons by activating ERK1/2 and subsequently, TGF-β2 signaling pathways. The effects of this neurotrophin are also likely attributable to non-hippocampal sources since BDNF expression in the early postnatal hippocampus is poor (Friedman *et al.*, 1991). While it remains to be seen whether the reported findings represent independent, interdependent, or parallel actions of BDNF and TGF-β2, our experiments identify two distally-produced growth factors, sharing common downstream signaling pathways that act in a temporally co-ordinated fashion to control neuronal proliferation and maturation.

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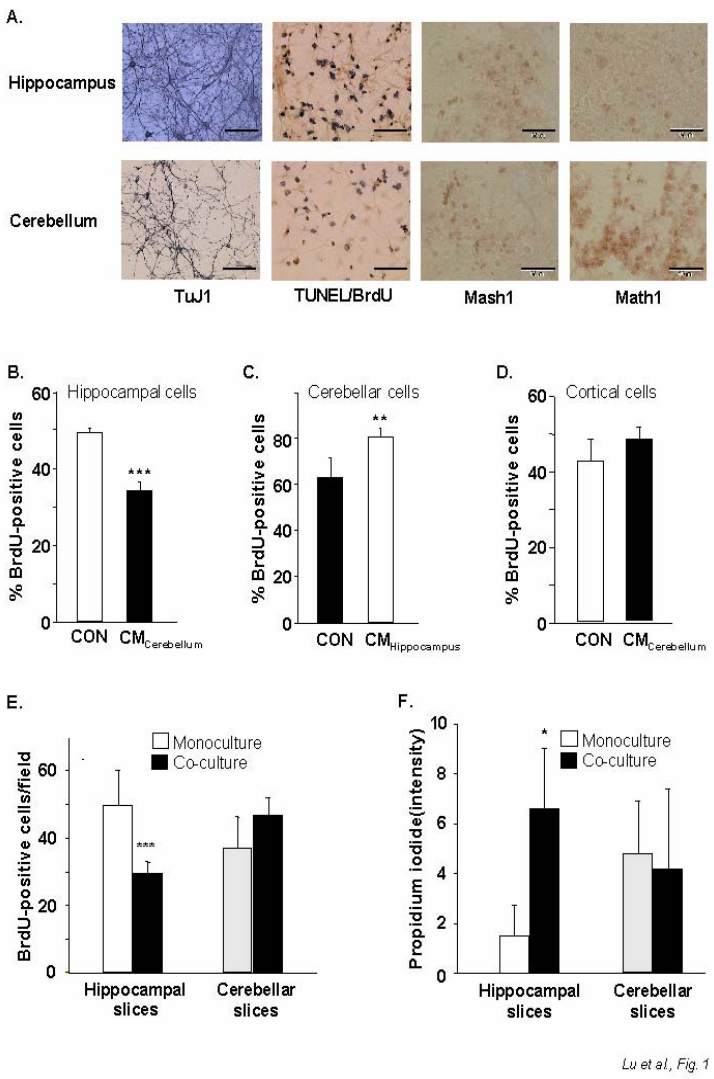



**Fig. 1. Cerebellar 'stop' *vs.* hippocampal 'go' signals.**

(A) Characteristics of hippocampal and cerebellar neuronal cultures under basal conditions, showing expression of the neuronal markers TuJ1, Mash1 and Math1, and levels of apoptosis (TUNEL) and proliferation (BrdU incorporation). Exposure of hippocampal cells to $CM_{Cerebellum}$ reduces BrdU incorporation (B); exposure of cerebellar cultures to $CM_{Hippocampus}$ stimulates BrdU uptake (C); $CM_{Cerebellum}$ does not influence proliferation in cortical neurons (D). In hippocampal-cerebellar slice co-cultures, hippocampal cell proliferation is reduced in the presence of cerebellar slices, whereas cerebellar cell proliferation is slightly increased in the presence of hippocampal tissue (E). Cell death (propidium iodide assay) is increased in hippocampal, but not cerebellar, cells in hippocampal-cerebellar slice co-cultures (F). Scale bars in (A) represent 50 μm. Here and in all subsequent figures, numerical data refer to means $\pm$ s.e.m. (n = 4-6). *, ** and *** indicate $P < 0.05$, 0.01 and 0.001, respectively.

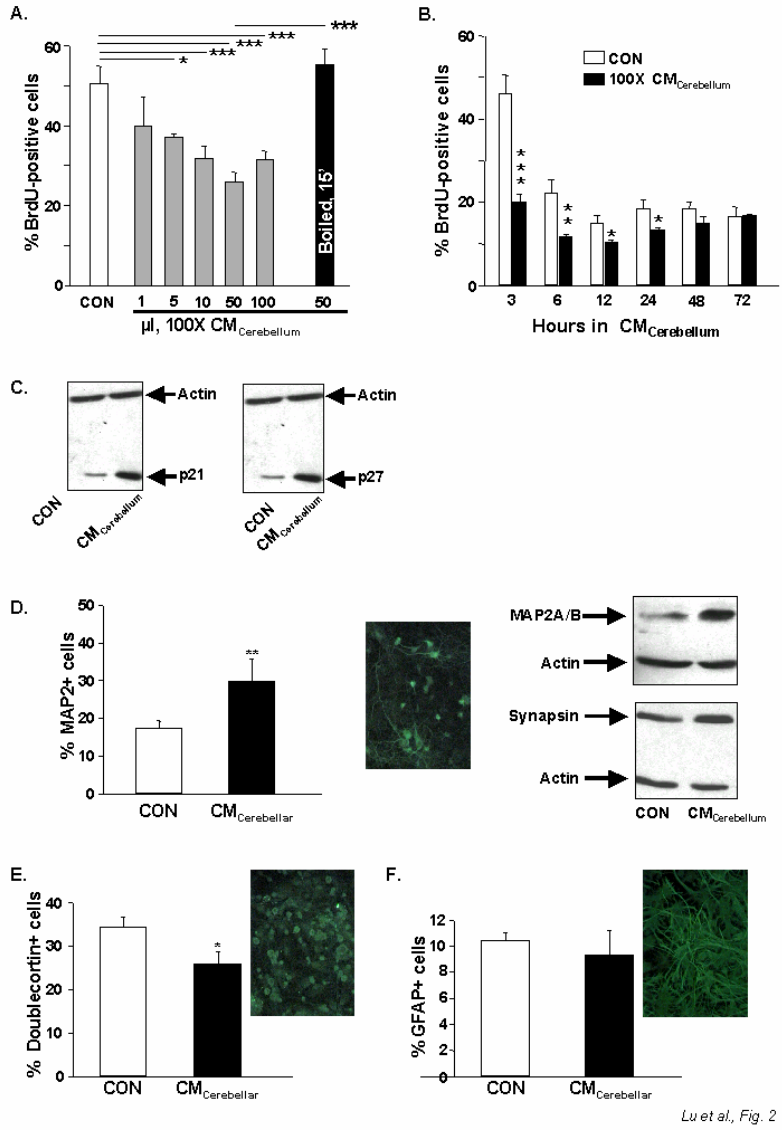



**Fig. 2. Hippocampal cultures express markers of cell cycle inhibition and neuronal differentiation after incubation in $CM_{Cerebellum}$.**

Dose- and time-dependency of $CM_{Cerebellum}$ anti-mitotic effects in hippocampal cultures (A,B); anti-proliferative activity of $CM_{Cerebellum}$ is abolished by boiling (A). Western blots and/or immunofluorescence showing that addition of $CM_{Cerebellum}$ to hippocampal cultures induces expression of the cyclin-dependent kinase inhibitors p21 and p27 (C) and of the mature neuron markers MAP2A/B and synapsin (D); concomitantly, expression of the neuroblast marker doublecortin is decreased (E). Proliferation of GFAP-positive glial cells is not affected by $CM_{Cerebellum}$ treatment (F).

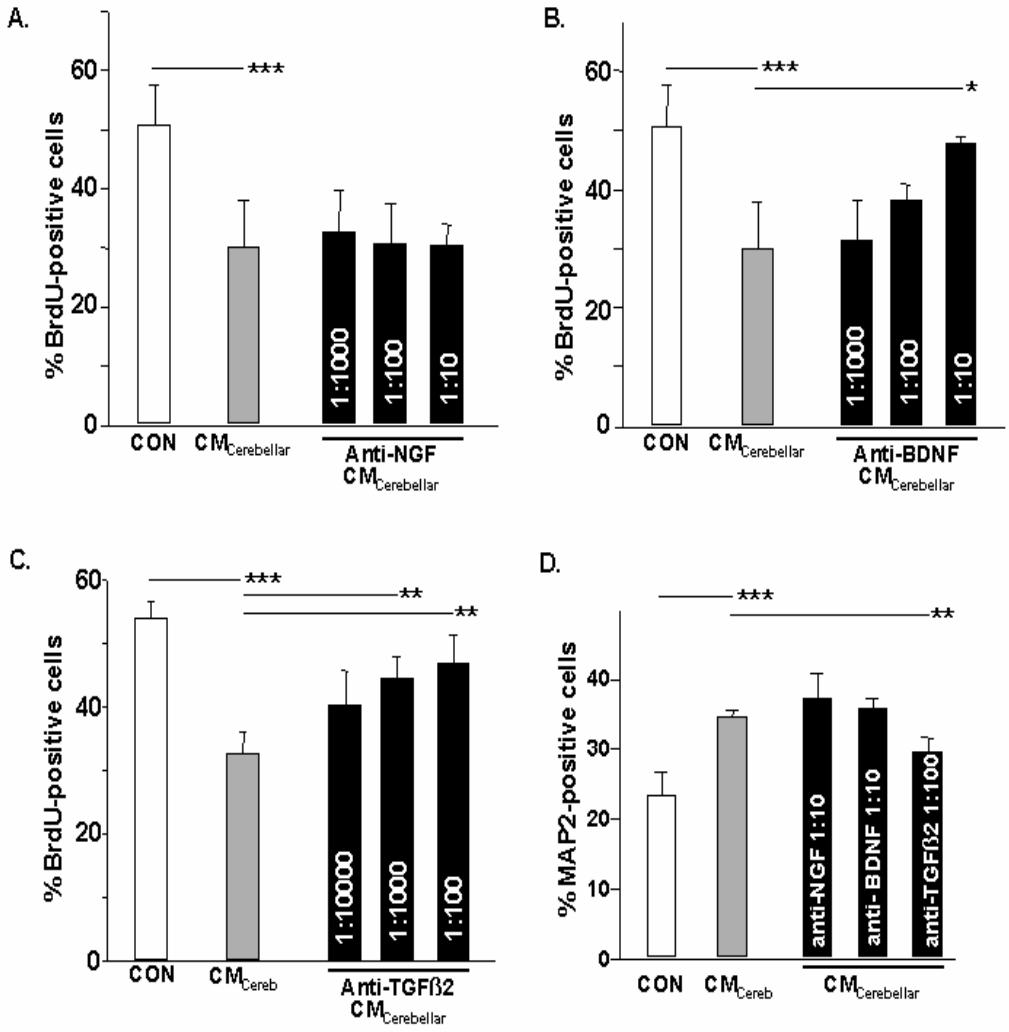



**Fig. 3. Immunoneutralization of candidate anti-proliferative and differentiating factors in $CM_{Cerebellum}$.** $CM_{Cerebellum}$ was preadsorbed with the indicated dilutions of anti-NGF (A), BDNF (B), or TGF-β2 (C,D). Immunoneutralization of BDNF and TGF-β2 significantly attenuated the anti-proliferative actions of $CM_{Cerebellum}$; anti-TGF-β2 significantly attenuated the pro-differentiating effects of $CM_{Cerebellum}$, assessed by MAP2A/B expression (D).

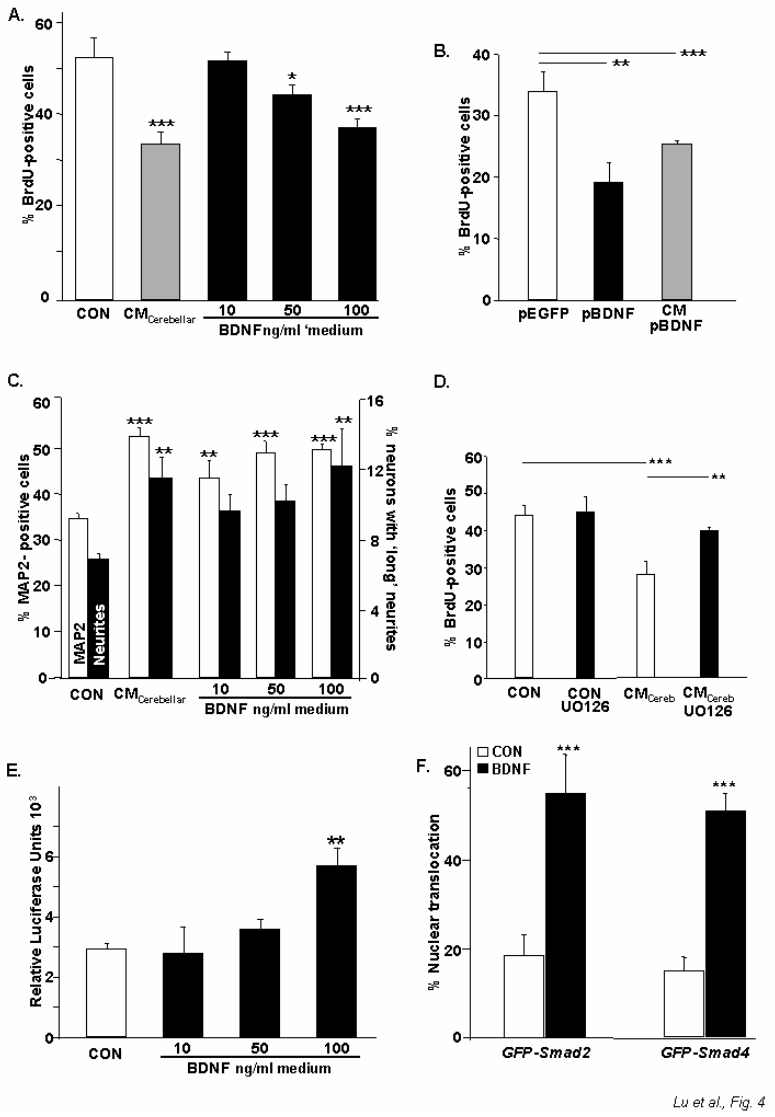



**Fig. 4. Exogenous BDNF replicates $CM_{Cerebellum}$ actions through the mediation of ERK1/2 and TGF-β2 signaling pathways.**

(A) BDNF peptide dose-dependently inhibits BrDU retention in hippocampal cells. (B) Comparison of the first two bars shows that hippocampal cells transiently transfected with pBDNF show reduced BrdU incorporation; control transfections were done with pEGFP. Inhibition of BrdU uptake is also seen in cells exposed to CM (24 hours) from cells transfected with pBDNF (third bar). (C) BDNF stimulates maturation of hippocampal cultures (increased expression of MAP2A/B and neurons with neurite lengths > two times the diameter of the soma). (D) The MEK1/2 inhibitor, UO126 (5 μM), counteracts the anti-proliferative effects of BDNF. (E) Increases in the transactivation of the TGF-β-responsive 3TP-Lux reporter gene by BDNF in primary hippocampal cells implicates mediation of BDNF actions by TGF-β signaling pathways. (F) Involvement of TGF-β signaling pathways in mediating BDNF actions are also indicated by the ability of BDNF to stimulate cytoplasm-to-nucleus translocation of Smad2 and Smad4 (cf. Fig. 5C). In this last experiment, cells were pretreated with cyclohexamide (10 μM, 1 hour) before addition of BDNF (1 hour) in order to ensure that any BDNF effects observed were not confounded by de novo synthesis of other intermediary factors.

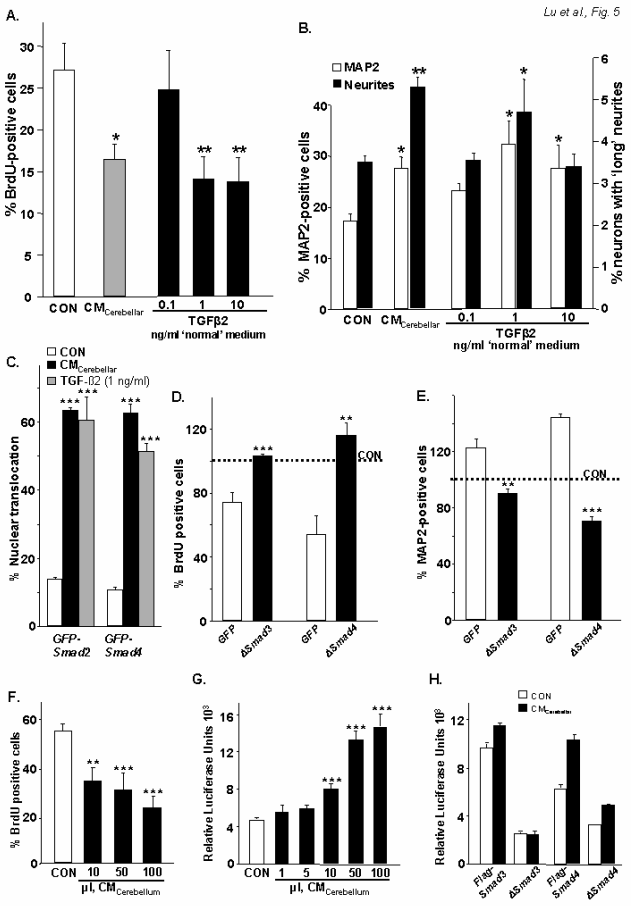



**Fig. 5. Involvement of TGF-β2 signaling pathways in the anti-mitotic and differentiating effects of of $CM_{Cerebellum}$.**

Exogenous TGF-β2 reproduces the anti-proliferative (A) and pro-differentiating (B) actions of $CM_{Cerebellum}$ in primary hippocampal cultures. Exposure of hippocampal cells to $CM_{Cerebellum}$ induces nuclear translocation of the TGF-β2-specific partner Smad2 and of Co-Smad4, as shown by transient transfection experiments (C); for comparison, nuclear translocation after TGF-β2 (1 ng/ml) is shown. Introduction of dominant negative forms (Δ) of either Smad3 which specifically couples with TGF-β2 or of Co-Smad4 abrogates the anti-mitotic (D) and differentiating (E) effects of $CM_{Cerebellum}$ on primary hippocampal cells. $CM_{Cerebellum}$ also exerts anti-proliferative effects on hippocamus-derived HiB5 cells (F). $CM_{Cerebellum}$ stimulates generation of luciferase from the 3TP-Lux reporter in HiB5 cells (G); the latter is abrogated when dominant negative forms (Δ) of either Smad3 or Co-Smad4 are co-expressed.

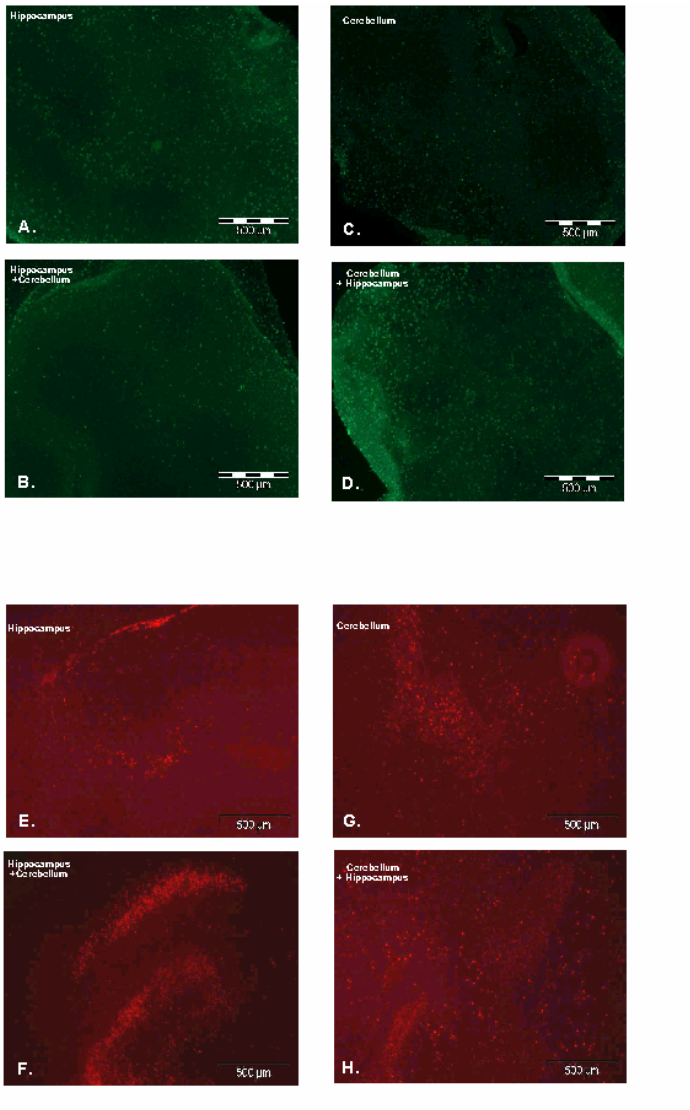
TABLE 1

**Multiple factors in $CM_{Cerebellum}$ contribute to its anti-proliferative, apoptotic and differentiation-inducing properties.**

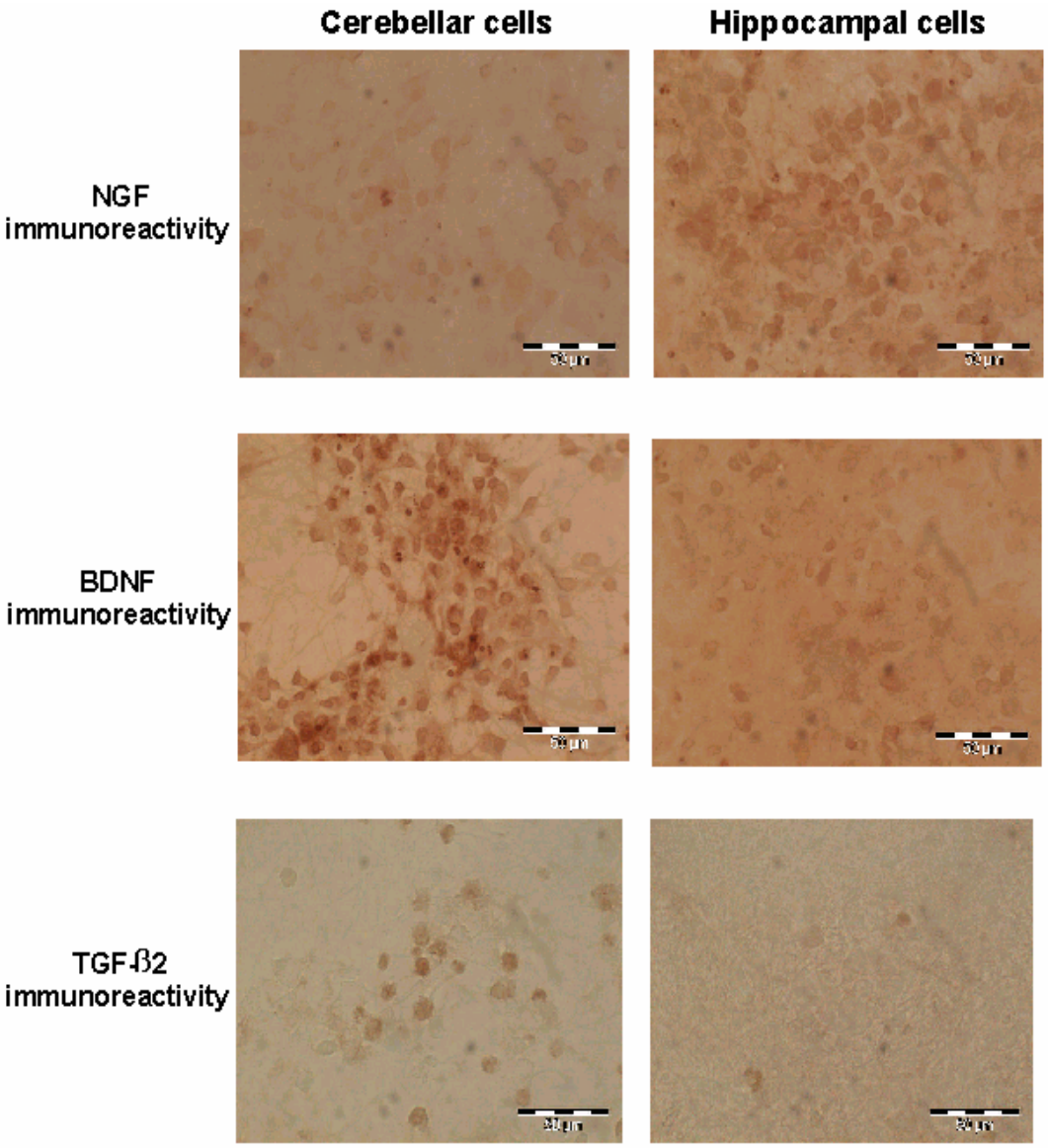
Concentrated $CM_{Cerebellum}$ was fractionated according to ionic strength and dye affinity using Q and Affigel Blue chromatography. The 4 fractions that eluted with NaCl (0.1-1.5 M) differentially influenced proliferation, apoptosis and differentiation in primary hippocampal cells. Specifically, fractions with 0.1 M NaCl displayed significant anti-mitotic ($p < 0.001$) and anti-apoptotic ($p < 0.05$) activities; strong anti-apoptotic activity was observed in fractions eluting with 1.5 M NaCl ($p < 0.001$) whereas those eluting with 1 M NaCl promoted neuronal maturation ($p < 0.05$).

| | Medium | Biological Activity | | |
|---|---|---|---|---|
| | | Proliferative (% $BrdU^+$ cells) | Apoptotic (% $TUNEL^+$ cells) | Differentiating (% $MAP2^+$ cells) |
| | Control | 45.1 ± 3.9 | 22.7 ± 1.3 | 15.2 ± 1.6 |
| | $CM_{Cerebellar}$ Crude | 13.5 + 1.2 | 27.1 + 1.5 | 26.4 + 0.5 |
| Q-ion exchange chromatograpgy $CM_{Cerebellum}$ | Eluate, 0.1 M NaCL | 18.5 ± 1.4 | 16.3 ± 1.2 | 16.5 ± 0.8 |
| | Eluate, 0.5 M NaCL | 38.1 ± 3.5 | 21.8 ± 2.2 | 13.4 ± 0.5 |
| | Eluate, 1 M NaCL | 46.9 ± 0.8 | 22.6 ± 4.1 | 22.2 ± 1.2 |
| | Eluate, 1.5 M NaCL | 46.5 ± 1.9 | 8.7 ± 1.3 | 19.2 ± 2.8 |

SUPPLEMENTARY FIGURES




**Supplementary Fig. 1. Proliferation and apoptosis in hippocampal-cerebellar slices co-cultures.**
Compared to hippocampal slice monocultures (A), hippocampal-cerebellar slice co-cultures (B) stained for BrdU show reduced proliferation in hippocampal slices. BrdU incorporation in cerebellar slices was not markedly altered by the presence of hippocampal slices (cf. C,D). Hippocampal slices co-cultured with cerebellar slices (F) showed stronger retention of propidium iodide, indicating increased apoptosis, as compared to hippocampal mono-cultures (E). Propidium iodide staining in cerebellar slices (G) was not altered after co-culture with cerebellar slices (H).



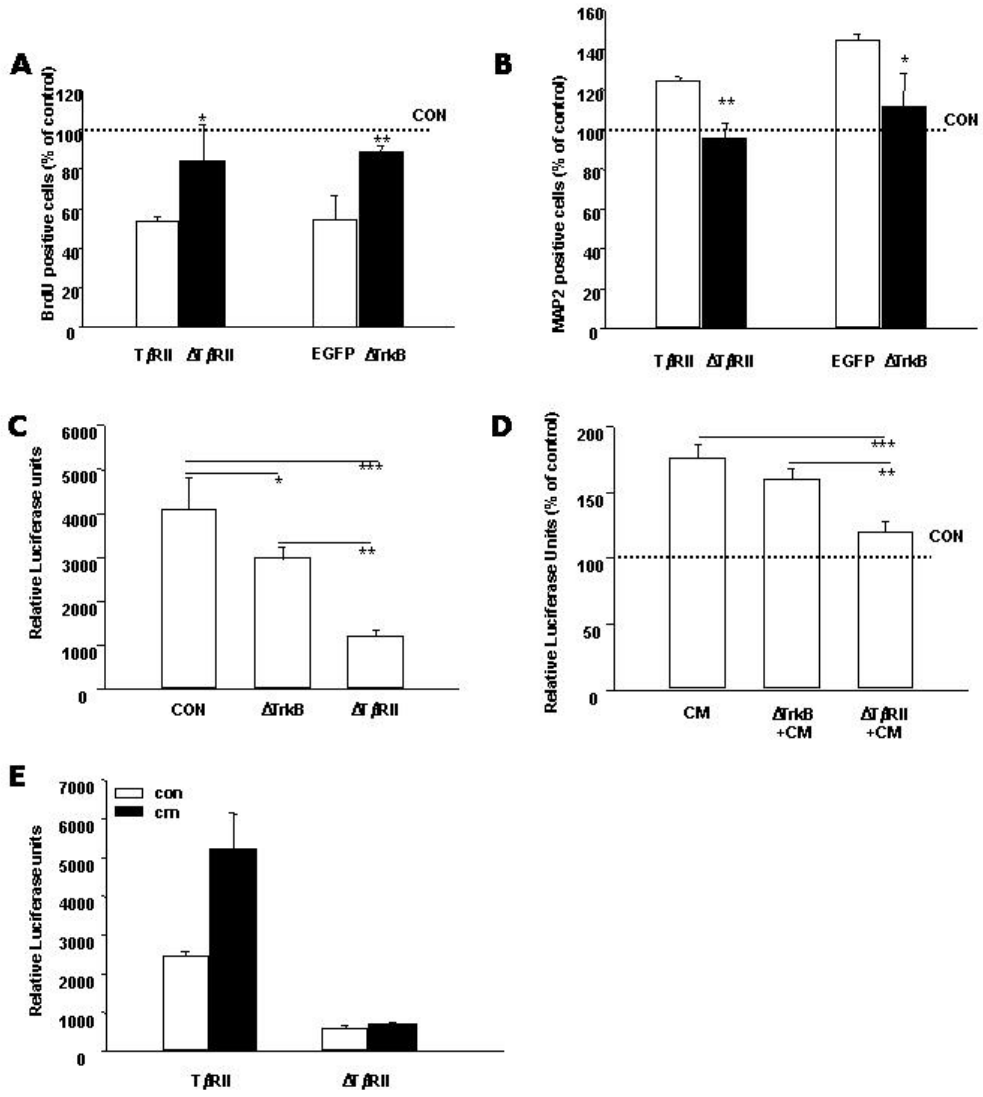

**Supplementary Fig. 2. Differential expression of NGF, BDNF and TGF-β2 in cerebellar and hippocampal primary cultures, demonstrated by immunocytochemistry.** Note the less intense staining of BDNF and TGF-β2 in hippocampal vs. cerebellar cultures.

## 2.3. Additional data
## Transfection studies and different *in vitro* periods
(Results obtained by the authors related to the present chapter)

### 2.3. Additional data

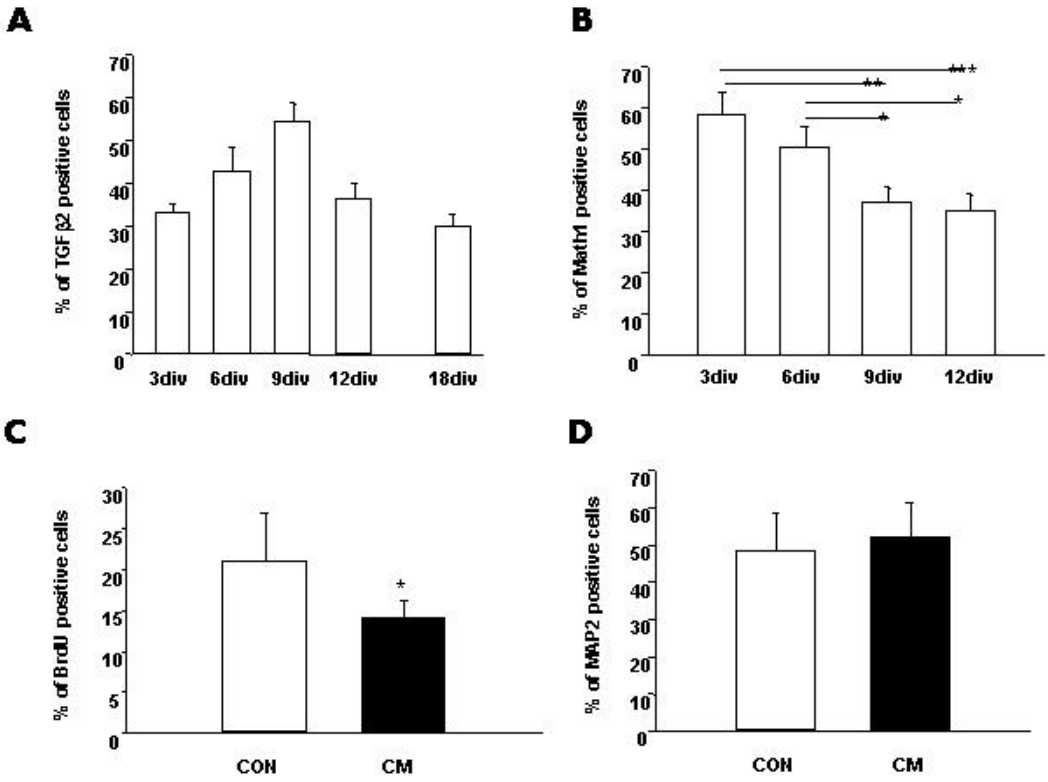
### Transfection studies and different *in vitro* periods

Results from transfection of cells with a dominant negative form of TGFβ receptor or 3TP-Luc and data from immunofluorescent staining of primary cerebellar cultures from P7 rats, maintained in vitro for various periods.




**A, B:** primary hippocampal cells; **C-E:** Hib5 cell line.

**A.** Transfection of cells with a dominant negative form of TGFβ receptor II (ΔTβRII) before treatment with $CM_{Cerebellum}$ resulted in a significant increase in proliferation, as compared to transfection with TGFβ receptor II (TβRII). Similarly, transfection with a dominant negative form of TrkB (ΔTrkB) stimulated BrdU incorporation after treatment with $CM_{Cerebellum;}$ in the latter experiment, EGFP was used as a transfection control. **B.** Results from measurements of MAP2 expression in cultures treated as described in **A**, show that inhibition of expression of TβRII or TrkB prevents $CM_{Cerebellum}$-induced neuronal differentiation. **C.** HiB5 cells were transfected with the 3TP-Luc (Smad pathway) reporter gene and either ΔTβRII or ΔTrkB. Under basal conditions, luciferase activity was inhibited significantly in the presence of both dominant negative constructs. **D.** In a similar experimental design to that shown in **C**, the ability of $CM_{Cerebellum}$ to transactivate 3TP-Luc in the presence of ΔTβRII is significantly reduced *vs.* control, whereas in the presence of ΔTrkB there is no significant effect as compared to control. **E.** Cells were transfected with 3TP-Luc and either TβRII or ΔTβRII before exposure to control or $CM_{Cerebellum}$; results again show that functional TβRII is necessary to see $CM_{Cerebellum}$ activation of the Smad pathway.




Results from immunofluorescent staining of primary cerebellar cultures from P7 rats, maintained *in vitro* for various periods. **A, B** show relative numbers of TGFβ2- and Math1-positive cells, respectively, during the course of culture. **C, D** show results of BrdU incorporation studies and MAP2 immunostaining in cultures (8 DIV) exposed for 24h to $CM_{Cerebellum}$ obtained from cerebellar cultures that were 19 DIV.

# Chapter 3

## Direct targeting of hippocampal neurons for apoptosis by glucocorticoids is reversible by mineralocorticoid receptor activation

3.1. Supplementary background information

3.2. Crochemore C., Lu J., Wu Y., Liposits Zs., Sousa N., Holsboer F. and Almeida O.F.X (2004). *Molecular Psychiatry* (Manuscript under review)

3.3. Additional data

### 3.1. Supplementary background information

Loss of neurons bearing GR and MR in hippocampus is considered to be a primary cause of disinhibited HPA activity. GR activation induces apoptosis of granule cells in the hippocampus. In contrast, neuroprotection is seen with MR activation (Hassan *et al.*, 1996; Almeida *et al.*, 2000; Macleod *et al.*, 2003). The opposing actions of MR and GR on neuronal survival result from their ability to differentially influence the expression of members of the Bcl-2 family of pro- and anti-apoptotic proteins. Specifically, in the rat hippocampus, activation of GR induces cell death by increasing the ratio of the proapoptotic molecule Bax relative to the antiapoptotic molecules Bcl-2 or Bcl-x(L); the opposite effect is observed after stimulation of MR. In addition, GR activation increases and MR activation decreases levels of the tumor suppressor protein p53 (a direct transcriptional regulator of bax and bcl-2 genes). Similar findings were obtained in neural cell cultures (Crochemore *et al.*, 2002).

*In vivo*, the hippocampus receives neural inputs from other cortical and subcortical structures, as well as humoral inputs from the periphery which, together complicate analysis of the underlying mechanism of glucocorticoid-induced cell death. Previous *in vivo* studies using the synthetic glucocorticoid dexamethasone (DEX) were questioned on the basis that because DEX treatment produces 'chemical adrenalectomy' (de Kloet *et al.*, 1998); adrenalectomy itself induces apoptosis (Sloviter *et al.*, 1993, Sousa *et al.*, 1997). Moreover, at least in mice, P-glycoproteins hinder the access of DEX to the brain (Schinkel *et al.*, 1995; Meijer *et al.*, 1998). Further, it was previously shown that aldosterone or low levels of corticosterone can prevent adrenalectomy-indiced apoptosis, probably by activating MR (Sloviter *et al.*, 1995, Woolley *et al.*, 1991).

The above-summarized problem was resolved in an *in vitro* model, free from all peripheral influences, namely primary hippocampal cell cultures. The results described demonstrate the inherent apoptosis-inducing properties of DEX which become better evident if the neuroprotective actions mediated by MR are blocked pharmacologically.

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3.2. Crochemore C., Lu J., Wu Y., Liposits Zs., Sousa N., Holsboer F. and Almeida O.F.X (2004). Direct targeting of hippocampal neurons for apoptosis by glucocorticoids is reversible by mineralocorticoid receptor activation. *Molecular Psychiatry*.

(Manuscript under review)

# Direct targeting of hippocampal neurons for apoptosis by glucocorticoids is reversible by mineralocorticoid receptor activation

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Key words: apoptosis, dexamethasone, glucocorticoid receptor, mineralocorticoid receptor, spironolactone, RU28318, hippocamus

Running title: Glucocorticoids and hippocampal cell death

*Abbreviations:*

| | |
|---|---|
| **ALDO** | aldosterone |
| **BSA** | bovine serum albumin |
| **DAB** | 3,3´-diaminobenzidine tetrahydrochloride |
| **DEX** | dexamethasone |
| **DIV** | days *in vitro* |
| **GAPDH** | glyceraldehyde-3-phosphate dehydrogenase |
| **GFAP** | glial fibrillary acidic protein |
| **GR** | glucocorticoid receptor |
| **Hoechst 33342** | Phenol, 4-[5-(4-methyl-1-piperazinyl)[2,5'-bi-1H-benzimidazol] -2'-yl]-, trihydrochloride |
| **MAP2** | microtubule-associated protein 2 |
| **MR** | mineralocorticoid receptor |
| **SPIRO** | spironolactone, [7 [alpha ] -(acetylthio)-3-oxo-17 [alpha ] -pregn-4-ene,21 carbolactone] |
| **RU28318** | Oxprenoate potassium , (7a,17a)-17-Hydroxy-3-oxo-7-propylpregn-4-ene-21-carboxylic acid, potassium salt |
| **TdT** | terminal deoxynucleotidyl transferase |
| **TUNEL** | TdT-mediated dUTP nick-end labeling |

## Abstract

An important question arising from previous observations *in vivo* is whether glucocorticoids can directly influence neuronal survival in the hippocampus. To this end, a primary postnatal hippocampal culture system containing mature neurons and expressing both glucocorticoid (GR) and mineralocorticoid (MR) receptors was developed. Results show that the GR agonist dexamethasone (DEX) targets neurons (MAP2-positive cells) for death through apoptosis. GR-mediated cell death was counteracted by the MR agonist aldosterone (ALDO). Antagonism of MR with spironolactone (SPIRO) causes a dose-dependent increase in neuronal apoptosis in the absence of DEX, indicating that nanomolar levels of corticosterone present in the culture medium which are sufficient to activate MR, can mask the apoptotic response to DEX. Indeed, both SPIRO and another MR antagonist, oxprenoate (RU28318), accentuated DEX-induced apoptosis. These results demonstrate that glucocorticoids can act directly to induce hippocampal neuronal death and that demonstration of their full apoptotic potency depends on abolition of survival-promoting actions mediated by MR.

## Introduction

Disorders of mood and cognition are linked to hypersecretion of glucocorticoids (GC) resulting from reduced efficacy of GC negative feedback.[1, 2, 3] One enduring view is that a loss of neurons bearing glucocorticoid (GR) and mineralocorticoid (MR) receptors in the hippocampus is the primary cause of disinhibited hypothalamo-pituiatry-adrenal (HPA) activity; moreover hippocampal cell loss is likely to have repercussions on cognition and the regulation of mood and anxiety.

Numerous previous experiments suggest that excessive corticosteroid secretion endangers the survival of hippocampal neurons by increasing their vulnerability to concomitant stimuli such as excitatory amino acids, calcium influxes and reactive oxygen species.[4, 5] The detrimental effects of corticosteroids on the hippocampus are backed by imaging studies in humans: there is a strong negative correlation between cortisol levels and hippocampal volume in patients with major depression.[6, 7] and in subjects with Cushing's disease;[8] reduced hippocampal volumes were also recently found in rats treated with dexamethasone (DEX), a GR agonist.[9] While most of the hippocampal shrinkage observed may be due to neuritic atrophy, including dendritic impoverishment and synaptic loss,[10, 11, 12], other experiments have demonstrated that GR activation activates a molecular cascade leading to significant levels of neuronal cell death through apoptotic mechanisms.[13] Besides apoptosis of mature hippocampal neurons,[14] there is also evidence that glucocorticoids interfere with proliferation granule neurons of the dentate gyrus.[15]

MR and GR both bind cortisol/corticosterone, albeit with differing affinities: whereas GR become occupied by corticosteroid levels in the high physiological range, MR appear to be tonically active under basal levels of HPA activity and there is evidence that activity of the two receptors is coordinated so as to maintain endocrinological and behavioral balance.[1, 16, 17] We previously proposed that the relative occupation of MR and GR also contributes to maintenance of hippocampal cell numbers, since activation of MR counteract deleterious glucocorticoid actions on neuronal survival.[11, 13, 14] Indeed, the results of earlier studies suggest that MR occupation may be essential for the survival of dentate granule neurons.[19, 20, 21]

The interpretation of previous data showing that DEX can induce neuronal cell death in animals has been questioned on the basis that because DEX poorly penetrates the brain and produces 'chemical adrenalectomy' by suppressing pituitary ACTH secretion, the observed cell loss may reflect abolition of MR activation rather than a direct effect of GR activation.[1] The work reported here re-addresses this issue by using a pharmacologically malleable model, namely primary hippocampal cell cultures. Our results demonstrate the inherent apoptosis-inducing properties of DEX which become better evident if neuroprotective actions mediated by MR are blocked.

## Materials and Methods

Experiments on animals were conducted in accordance with the European Communities Council Directive No. 86/609/EEC and local regulations. Hippocampal tissue was obtained from male rat pups, aged 4-5 days (Wistar rats; Charles River, Sulzfeld, Germany). Pilot *in vivo* studies in rats of this age showed an acute injection of DEX (100 µg/kg, i.p.) to result in a 60% increase in apoptotic cells in the hippocampus (measured by TUNEL histochemistry) within 24 h.

Tissue and cell dissociation: Rat pups were rapidly decapitated their brains carefully dissected out and placed in ice-cold Neurobasal/B27 solution (Invitrogen, Karlsruhe, Germany). Hippocampi were dissected out and freed from meninges and vascular tissue excess under a dissecting microscope, before being sliced (ca. 250 µm thick) on a McIlwain tissue-chopper. Slices were briefly washed in ice-cold Neurobasal/B27 medium, centrifuged briefly (70 g; 20° C) and resuspended in a solution containing papain; to this end, the Papain Dissociation Kit (Worthington Biochemical Corp., Freehold, NJ, USA) was used with some minor modifications to the manufacturer's suggested protocol (see ref. 22). Tissue digests were triturated gently and transferred to a medium consisting of Neurobasal/B27 medium, 1% fetal calf serum and 0.2% bovine serum albumin (BSA) at 37° C, filtered through a sterile nylon mesh (30 µm pore-size) and centrifuged at 200 g (20° C; 5 min.) before resuspension in Neurobasal/B27 medium containing enzyme inhibitor (ovomucoid/0.005% DNAse; Worthington). Aliquots of this suspension were run through a 1-step ovomucoid/BSA density gradient (centrifugation at 70 g at 20° C for 5 min.), taken up in Neurobasal/B27 medium containing basic fibroblast growth factor (bFGF) (10ng/ml), Glutamax I (0.5mM) and kanamycin (100µg/ml) (all from Invitrogen) and plated on gelatin/PDL-coated glass coverslips (Superior, Bad Mergentheim, Germany) at a density of 450 $\pm$ 33 cells/mm$^2$. Cultures were maintained at 37° C, under 5% $CO_2$ and 99% relative humidity.

Treatments: Cells were exposed to drugs after 6 days *in vitro*; exposure to experimental drugs was for 48 h. The GR agonist (DEX) was obtained in aqueous form (Fortecortin®, Merk, Darmstadt, Germany) and used at doses of either $10^{-6}$ M or $10^{-5}$ M. The prototypic MR agonist aldosterone (ALDO) was purchased from Sigma (Deisenhofen, Germany) and used at a dose of $10^{-5}$ M (survival studies) or $10^{-8}$ M (translocation experiments) after solution in ethanol. Two MR antagonists were used alone or as co-treatments with DEX: spironolactone (SPIRO; Sigma) was used at between $10^{-9}$ and $10^{-5}$ M after solution in ethanol; oxprenoate (RU28318), purchased from Tocris Cookson (Bristol, UK), was used at dose ranging from $10^{-9}$-$10^{-5}$ M after direct solution in growing medium. Final ethanol concentrations in medium were 0.01%. In all experiments, the position of wells subjected to control or drug treatments was randomized; each experiment was performed on at least 3 independent occasions.

*Hoechst staining*: The total number of cells per unit area was determined by counting fluorescent nuclei (Hoechst-stained). Briefly, cells were fixed in ice-cold 4% paraformaldehyde (10 min.), washed in PBS and incubated with Hoechst 33342 (1 µg/ml; Molecular Probes, Leiden, The Netherlands) for 45 min. in the dark. After rigorous washing (PBS), coverslips were mounted in anti-fading medium before counting using the cell counting parameters described below.

*Immunocytochemistry:* For characterization of cultures by light microscopy, cells were fixed in ice-cold 4% paraformaldehyde (10 min.) and incubatzed with the following primary antibodies: mouse anti-microtubule-associated protein 2 (MAP2; Roche, Mannheim, Germany; 2 µg/ml), mouse anti-myelin basic protein for oligodendrocytes (MBP; Chemicon, Temecula, CA; 1:500) and rabbit anti-glial fibrillary acidic protein (GFAP; DAKO, Hamburg, Germany; 1:500). Peroxidase-conjugated antibodies (Sigma) and 3,3´-diaminobenzidine tetrahydrochloride (DAB; 0.025%; Sigma) were used to visualize immunoreactive elements. Specimens were examined on an Olympus BX-60 microscope, video-linked to a computer equipped with

image-processing software (ImagePro, Maryland, USA). Cell counts were performed on 10 randomly-chosen, equally-sized microscopic fields from 5 coverslips under 400X magnification. Data representing numbers of immunolabeled cells are expressed relative to total number of cells labeled with Hoechst dye (see above).

*Electron microscopic analysis:* For classical electron microscopic characterization of the cultures, cells were fixed in 1% paraformaldehyde/1% glutaraldehyde in 0.1 M phosphate buffered saline (PBS; pH 7.4) for 1 h. Thereafter, the cells were treated with 1% osmium tetroxide, dehydrated in a graded series of ethanol and flat-embedded in Epon resin.[23] Serial ultrathin sections were cut on an ultratome and contrasted with uranyl acetate and Reynold's lead citrate. Ultrastructural evaluation was carried out with a Hitachi transmission electron microscope.

*Detection of apoptosis by TUNEL histochemistry:* TdT-mediated dUTP nick end labeling (TUNEL) histochemistry was performed on 4% paraformaldehyde-fixed cells, as described.[14, 24] TdT was purchased from MBI Fermentas (Heidelberg, Germany). The above-detailed morphometric procedures were use to quantify numbers of apoptotic (TUNEL-positive) as a proportion of Hoechst-stained cells.

*Reverse-transcriptase PCR:* To detect *GR and MR* mRNA transcripts in the cultures, total RNA, free from chromosomal DNA contamination, was isolated (RNAeasy kit, Qiagen, Hilden, Germany) and reverse transcribed with SUPERSCRIPT$^{TM}$ II RNA H-reverse transcriptase (Life Technologies) using custom-synthesized oligo-$dT_{12\text{-}18}$ primers (MWG Biotech, Ebersberg, Germany). Published primers[25] were used and amplication conditions were denaturation for 40 s at 94 C, annealing for 1 min. at 62° C (*MR*) or 40° C (*GR*), and primer extension for 2 min. at 72° C; optimal amplifications of *MR* and *GR* mRNA were achieved after 35 and 25 cycles, respectively.

*Nuclear translocation of MR and GR:* After 6 days in vitro, cells were exposed to either DEX or ALDO (both at $10^{-8}$ M) for 6 h. Cells were then either prepared for detection of MR and GR by immunofluorescence (fixation as before) or Western blotting, using rabbit anti-glucocorticoid receptor (GR; Santa Cruz Biotechnology, Heidelberg, Germany; 1:1,500) or rabbit anti-mineralocorticoid receptor (MR; Santa Cruz; 1:1,500). Fluorescence images from DEX- or ALDO-treated cells were compared to those from non-treated cells; the prediction was that receptor immunoreactivity in the treated cells would be predominantly localized to cell nuclei. For Western blots, cells were lysed and processed as described previously.[26]

*Statistics*: All numerical data, shown as means $\pm$ SEM, was subjected to ANOVA and appropriate *post-hoc* analyses (SigmaStat 3). The level of significance was preset at $P \leq 0.05$.

## Results

### Model validation

Most previous investigations on corticosteroid effects on hippocampal cell survival have been performed in adult rats or on embryonic rat hippocampal cell cultures; in the present experiments, hippocampal cell cultures were prepared from postnatal rats, aged 4-5 days. Preliminary *in vivo studies* showed that the hippocampi of rats of this age are sensitive to the apoptosis-inducing actions of DEX, showing a 60% increase in cell death following an acute injection of the drug (data not shown).

Cells established dense networks *in vitro,* with some 25-30% of cells staining positively for MAP-2, a marker of mature neurons (Fig. 1a, b) as well as for GFAP (astrocytes; Fig. 1c) and myelin basic protein (oligodendrocytes; Fig. 1d). Electron microscopic analysis of the cultures revealed cells that displayed morphological features typical of neurons (Figs. 1e-j).

**Comment:** What's the % of these? And oligodendrocytes?

**Comment:** Ossie, I think we need more detail in the description of these. Figues i) and j) represent astrocytes, not neurons.

Consistent with results from *in vivo* studies,[27, 28] cells in culture were seen to express *MR* and *GR* mRNA (detected by RT-PCR analysis; Fig. 2a) as well as receptor protein (immunofluorescence images in Figs. 2b, c). Under basal conditions, MR were localized in both the cytoplasmic and nuclear compartments, probably owing to the low levels of cortisol in the medium supplement (B27); treatment with ALDO resulted in a greater intensity of immunoreactive signal in the nucleus. In contrast, GR in untreated cells were predominantly located in the cytoplasm, and were only found in the nucleus after exposure to DEX. The morphological localization of GR was corroborated by evidence obtained by Western blot analysis of cytoplasmic and nuclear fractions; Fig. 2d shows that DEX treatment results in a conspicuous re-location of GR from the cytoplasmic to the nuclear compartment. Together these data indicate that the receptors expressed *in vitro* are likely to serve their roles as ligand-activated transcription factors.

### DEX induces neuronal cell death in an ALDO-reversible manner

Treatment of hippocampal cultures with the GR agonist DEX ($10^{-5}$ M) doubled the relative number of TUNEL-positive (apoptotic) cells within 48 h ($P < 0.05$; Fig. 3a). A significant proportion of these apoptotic cells were mature neurons, as judged by the reduction in the MAP-2 immunoreactive sub-population ($P < 0.05$; Fig. 3b). Whereas exposure to ALDO ($10^{-5}$ M) did not affect the incidence of apoptosis or the survival of mature neurons, the MR agonist significantly attenuated the apotosis-inducing actions of DEX ($P < 0.5$; Fig. 3). Thus, consistent with our previous findings,[13, 14] MR occupation counteracts the actions GR.

**Comment:** This might be due to:
1- special vulnerability of neurons to DEX.
2- Alteration in neuronal differentiation? – less likely
Can we explore this more, e.g. by having the % of GFAP and MBP cells?

### MR antagonism accentuates apoptotic actions of DEX

Blockade of MR with spironolactone (SPIRO; $10^{-8}$-$10^{-5}$ M), resulted in a dose-dependent increase in levels of apoptosis (Fig. 4a). The less-potent MR antagonist oxprenoate (RU28318)[29, 30, 31] only stimulated apoptosis when used at $10^{-5}$M, the highest doses tested (Fig. 4b). The apoptotic actions of SPIRO and RU28318 most probably result from their counteraction of the pro-survival effects of the nanomolar (MR-activating) levels of corticosterone in the B27/Neurobasal medium; these results indicate that tonic occupation of MR is essential for neuronal survival. The view that MR activation promotes cell survival was boosted by the finding that both MR antagonists synergized with a sub-optimal dose of DEX ($10^{-6}$ M; 10-fold lower than the dose used in the previous experiment) to induce apoptosis; our observations show that manifestation of the full apoptotic potential of GR agonists *in vitro* can be masked by MR agonists that are inadvertently present in the medium.

## Discussion

While a number of potential molecular mechanisms may be proposed to explain the basis of impaired glucocorticoid feedback in the hippocampus, the impact of glucocorticoid-induced destruction of the neural substrate mediating this feedback has been a recurring theme in psychoneuroendocrinology over the last decade. A series of *in vivo* studies in rats previously demonstrated that the GR agonist DEX stimulates cell death in the hippocampus as well as in other selected brain regions such as the striatum and substania nigra.[13, 14, 32, 33] However, it remains unclear as to whether these effects occur directly or whether gluocorticoids merely exacerbate the neurotoxic effects of other more potent insults such as excitatory amino acids and reactive oxygen species.[34, 35, 36]

Direct neural actions of DEX have also been questioned on the basis of data showing that DEX has limited access to the brain.[1] Observations that adrenalectomy leads to apoptosis in the hippocampus,[19, 20, 37] together with the fact that DEX treatment leads to a state of 'chemical adrenalectomy',[1] boost the argument against direct effects of DEX on hippocampal cell survival. On the other hand, significant levels of apoptosis can be observed within 24 hours of a single injection of DEX,[14] whereas the effects of adrenalectomy display different temporal and spatial dynamics.[37, 38, 39] Further, the apoptotic effects of DEX were shown to be blocked with a GR antagonist;[32] GR are transcription factors and we previously that their activation by DEX can trigger a molecular death cascade in the hippocampus and cell cycle arrest in a neural cell line.[13, 26]

The issue of whether DEX acts directly on neurons to stimulate their demise cannot be resolved easily *in vivo*. We therefore attempted to address this question in primary neuronal cultures derived from early postnatal rats. These cultures were carefully characterized and their suitability for our studies verified, especially with respect to ontogeny of elements involved in the regulation and responsiveness of the HPA axis,[27, 30, 41] as well as to age-related differences in the *in vivo* responses to the cell death-inducing actions of corticosteroids.[14, 42] Exposure of cultures to DEX at a dose of $10^{-5}$ M led to a significant loss of mature (MAP-2 positive) neurons, an event accompanied by a significant increase in the incidence of apoptosis. The results obtained in this isolated hippocampal neuron model, in which DEX can directly access individual cells, therefore show that DEX has the intrinsic potential to induce neuronal cell death, and that its effects occur independently of the HPA axis and other confounding factors.

The apoptotic actions of DEX ($10^{-5}$ M) were shown to be significantly attenuated when the MR agonist ALDO ($10^{-5}$ M) was added to the culture medium. This finding is consistent with previous results obtained in rats.[11, 13, 14] Since ALDO on its own did not alter apoptotic cell and neuronal numbers, our results add currency to the view that MR can trigger neuroprotective mechanisms; the latter concept emerged from a number of older studies which showed that either ALDO or low, MR-activating levels of corticosterone could prevent or reverse adrenalectomy-induced apoptosis.[18, 19, 39] Notably, the effects of adrenalectomy cannot be ameliorated by the administration of DEX.[20, 43]

Chemically-defined media of the sort used in these studies have significant advantages over serum-containing media. Nevertheless, our experiments were somewhat confounded by the presence of corticosterone (nanomolar range, sufficient to activate MR) in the medium. This factor most probably accounts for our observation that the MR antagonist SPIRO increased neuronal cell death in a dose-dependent ($10^{-7}$-$10^{-5}$ M) fashion, although another (weaker) MR antagonist (RU28318) did not alter basal levels of apoptosis. Thehis

finding supports the interpretation that tonic activation of MR is essential for the survival of hippocampal neurons.

In view of the above results, we hypothesized that blockade of 'medium-activated' MR would accentuate the effects of GR stimulation; the hypothesis was proven correct by analysis of apoptosis after concomitantly treating cultures with either SPIRO or DEX and a sub-optimal dose of DEX: whereas DEX at a dose of $10^{-6}$ M did not induce cell death, its combination with a range of doses of SPIRO or RU28318 ($10^{-8}$-$10^{-5}$ M) significantly exacerbated the occurrence of neuronal apoptosis. Besides acting on MR, SPIRO can also antagonize androgen and progesterone receptors;[44] however, since similar results were obtained with RU28318, a drug with greater selectivity for MR, our inference that neuroprotective effects are mediated by MR is warranted.

In summary, evidence gained in a cellular model shows that glucocorticoids can lead to hippocampal cell death without the participation of other aggravating factors, so long as their effects are not masked by previously activated MR. The presented results also bolster the view that hippocampal neuronal survival depends on the tonic occupation of MR. Further, insofar that these data demonstrate the opposing roles of MR and GR in hippocampal cell survival, they add credibility to the 'receptor balance hypothesis' that was generated from endocrinological and behavioral studies.[1, 17] If glucocortiocid-induced hippocampal cell losses do indeed contribute to impaired regulation of the HPA axis, eventually leading to mood, anxiety and cognitive impairments, therapeutic tools designed to selectively activate MR or to specifically improve their signaling efficiency in neurons[45, 46] would be a worthwhile strategy.

**Acknowledgments**

We thank Rosa Buric, Julia Deicke and Dieter Fischer for technical assistance. C.C., J.L., and Y.W. were supported by fellowships from the Max Planck Society. The work was partly supported by the German Academic Exchange Service/Magyar Ösztöndij Bizottság (DAAD 323-PPP Ungarn), Acções Integradas Luso-Alemãs (DAAD-CRUP-ICCTI 314/Al-p-dr), and the European Commission (QLG3-CT-2000-00844).

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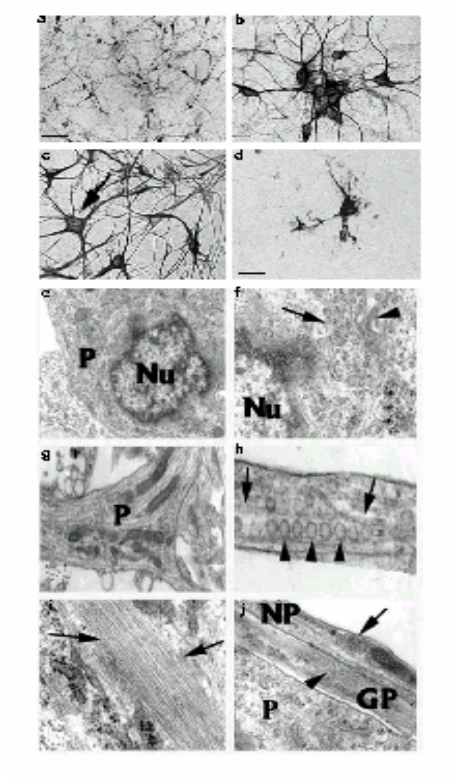



**Figure 1. Immunocytochemical and ultrastructural characterization of primary postnatal rat hippocampal cultures.**

(a) Low magnification field view of primary rat hippocampal cultures (5 days *in vitro*), immunostained with microtubule-associated protein 2 (MAP2). (b) High power micrography showing differentiated MAP2-immunolabeled neurons. (c) High-power micrograph of astrocytes immunostained for glial fibrillary protein (GFAP). (d) An immunoreactive oligodendrocyte labeled with anti-myelin basic protein. *Scale bars represent 120 µm* (a) *and 30 µm* (b-d)*.* (e) Medium-power electron micrograph of a cultured cell showing typical features of a neuron. Note the large nucleus (Nu) within the perikaryon (P) richly endowed with free and membrane–bound ribosomes. *(X10,000)*. (f) Electron micrograph showing numerous ribosomes (arrow) and cysts of a Golgi-complex (arrowhead) within the neuronal cytoplasm *(X28,000)*. (g) Demonstration of a growing neuronal process (P) exhibiting bundles of microtubules and mitochondria *(X 13,000)*. (h) High-power view of a neuronal process with microtubules organized in a parallel fashion (arrow). Note the association of ovoid transit vesicles (arrowheads) with microtubules *(X32,000)*. (i) High power micrograph of an astrocyte with a prominent bundle of glial filaments within its cytoplasm (arrows) *(X40,000)*. (j) Example of a neuronal perikaryon (P) lying adjacent to a glial process (GP) showing glial filaments (arrowhead). Also shown is a neuronal process (NP) growing along the surface of the glial element. The arrow points to a synaptic vesicle-sized element *(X30,000)*.

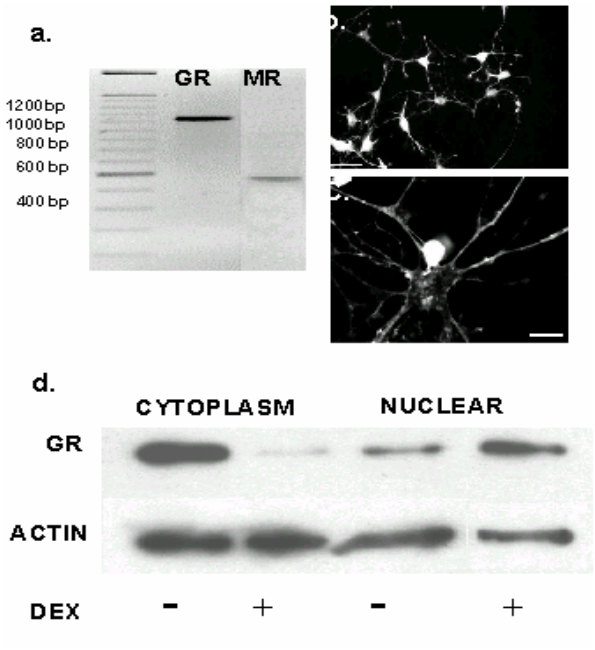



**Figure 2. Glucocorticoid (GR) and mineralocorticoid (MR) receptor expression and translocation in hippocampal cell cultures. (a)** RT-PCR demonstration of MR and GR expression in primary hippocampal cultures maintained for 5 days *in vitro*. The transcript size of each receptor corresponded with previously-published data.[25] **(b)** Low-power photomicrograph showing neurons displaying GR immunoreactivity. Note that the cells were treated with DEX ($10^{-7}$ M) for 6 h before fixation, and that the GR signal is mainly located in nuclei, indicating translocation of the receptor from its normal cytoplasmic (unliganded) location. (Scale bar represents 60 μm). **(c)** High power view of a neuron displaying MR-immunoreactivity in its nucleus following exposure to the prototypic MR agonist aldosterone ($10^{-8}$ M) for 6 h. (Scale bar represents 15 μm). Note that a small proportion of neurons and glia displayed MR immunoreactivity in their cytoplasma in the absence of exogenous ligand (data not shown), probably owing to the low concentrations of cortisol present in the growing (B27) medium. **(d)** Further demonstration (Western blot) that DEX treatment ($10^{-6}$ M) mobilizes immunoreactive GR to the nucleus.

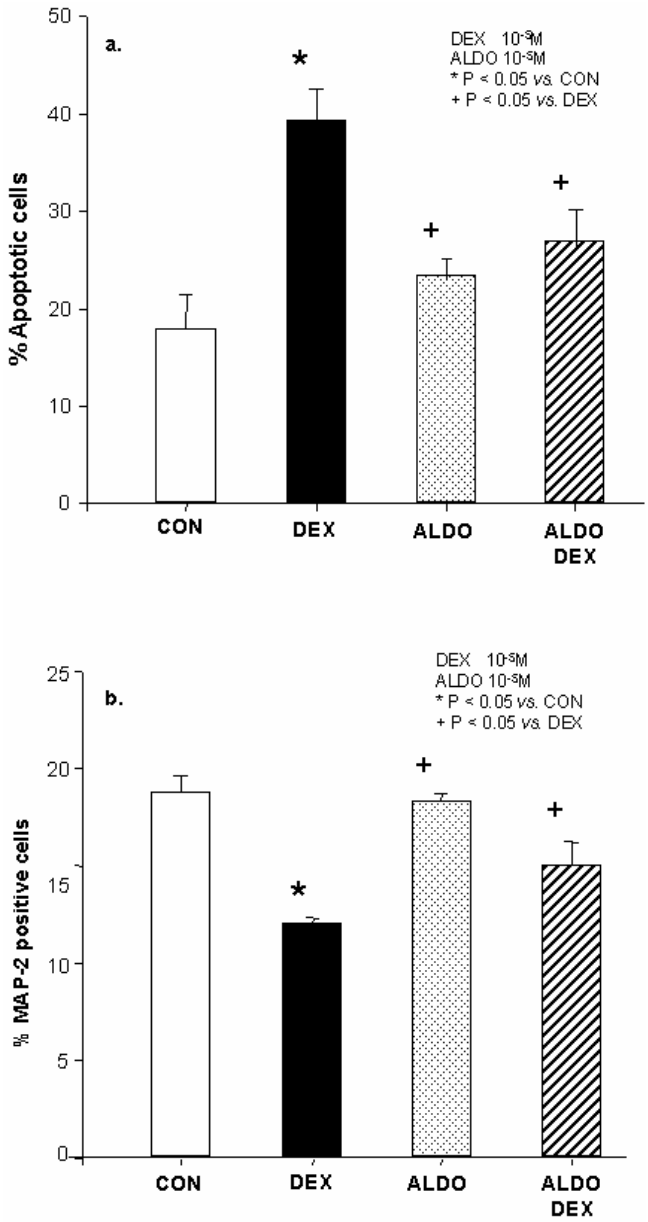



**Figure 3. Opposing effects of GR and MR ligands on hippocampal neuron survival. (a)** In contrast the effects of the MR agonist aldosterone (ALDO;$10^{-5}$ M), the GR ligand dexamethasone (DEX; $10^{-5}$ M) induces significant levels of apoptosis in hippocampal cultures. The apoptotic potential of DEX is significantly attenuated when cultures are concomitantly treated with ALDO and DEX. **(b)** The size of the neuronal (MAP-2 positive) subpopulation of cells in the cultures is significantly reduced in the presence of DEX ($10^{-5}$ M) and unaffected by ALDO ($10^{-5}$ M); the number of MAP-2 positive neurons is not significantly different from that observed in controls when cultures are exposed both ALDO and DEX. It is inferred from the mirror images of the data shown in (**a**) and (**b**) that DEX targets neurons for apoptotic death in an ALDO-reversible manner.

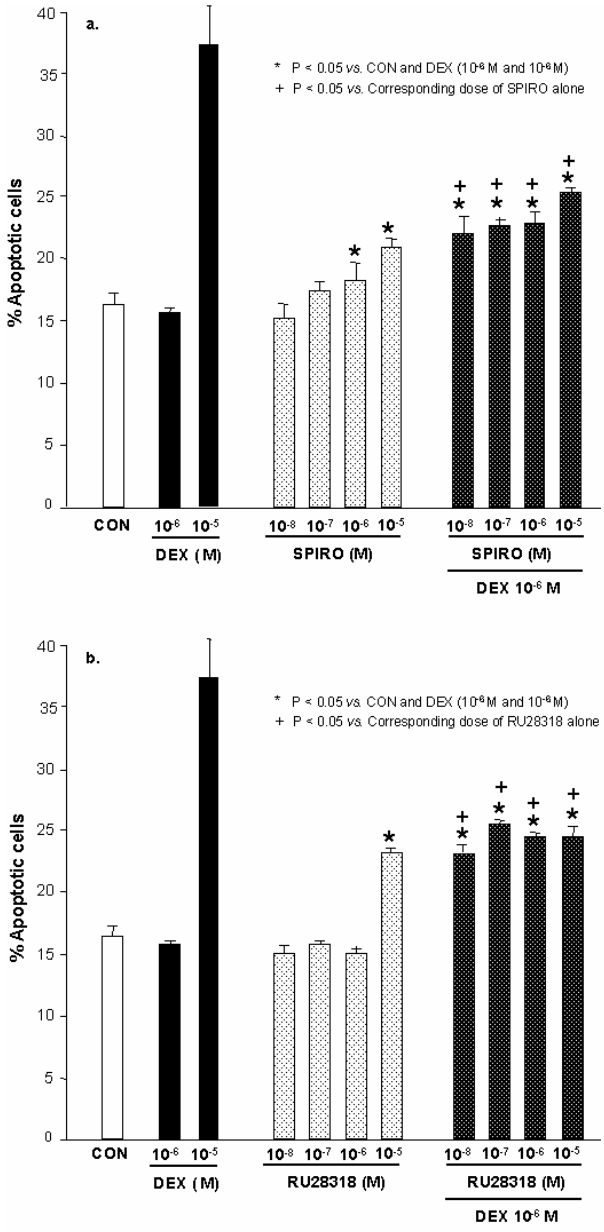



**Figure 4. Blockade of MR compromises neuronal survival and predisposes neurons to DEX-induced apoptosis.** As compared to the previously used dose of dexamethasone (DEX;$10^{-5}$ M; Fig. 3), a ten-fold lower dose of the GR agonist does not cause significant apotosis in hippocampal cultures (cf. second and third bars in **a** and **b**). Reasoning that this may have resulted from nanomolar (MR-activating) concentrations of levels of corticosterone in the culture medium, cells were exposed to two MR antagonists, spironolactone (SPIRO; **a**) and RU28318 (**b**); these compounds display slightly different pharmacological profiles.[29, 30, 31] In the absence of dexamethasone (DEX), addition of SPIRO ($10^{-8}$-$10^{-5}$ M) dose-dependently increased the incidence of apoptosis (**a**); RU28318 at a dose of $10^{-5}$ M also significantly stimulated apoptosis (**b**). In combination with the apoptosis non-inducing dose of DEX ($10^{-6}$ M; **a** and **b**), all doses of SPIRO and RU28318 led to levels of cell death that were significantly greater than those seen after treatment with the antagonist alone, i.e. blockade of MR with either SPIRO or RU28318 increased neuronal sensitivity to the apoptotic actions of DEX.

## 3.3. Additional data

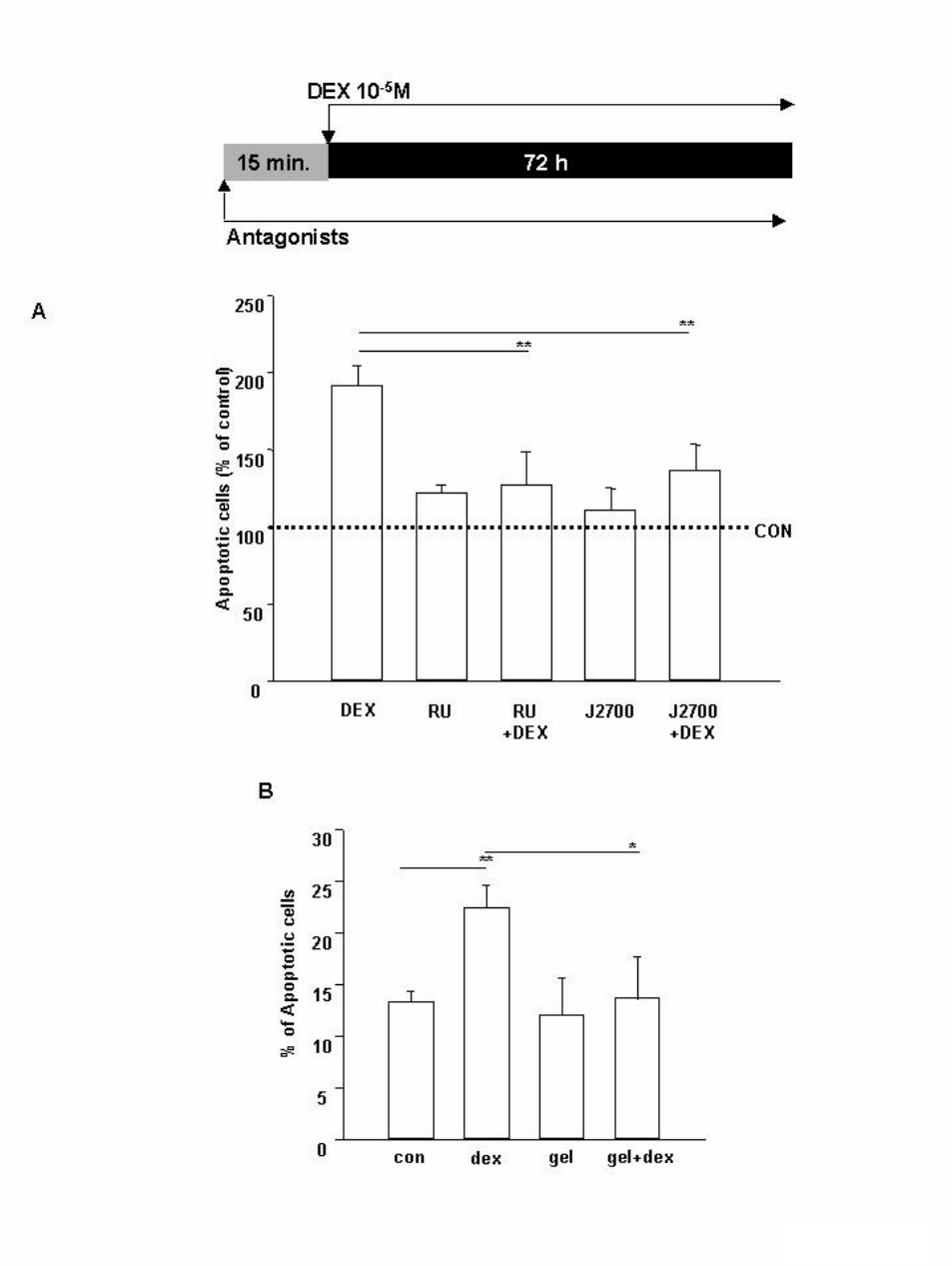
## Glucocorticoid-induced neuronal apoptosis: sparing of immature neurons and sacrifice of mature neurons

(Results obtained by the authors related to the present chapter)

### 3.3. Additional data

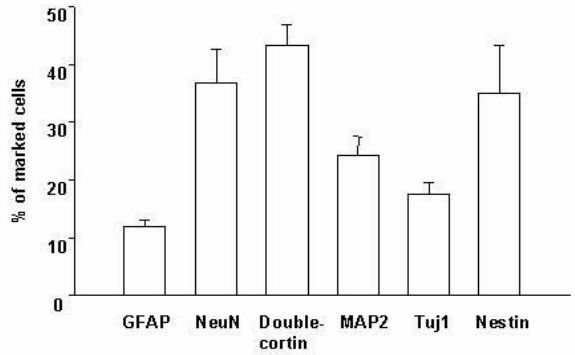
#### Glucocorticoid-induced neuronal apoptosis: sparing of immature neurons and sacrifice of mature neurons

Immature and mature (post-mitotic) neurons can be distinguished using immunochemical markers. Anti-nestin labels neuronal prercursors, whereas anti-doublecortin (DCX) labels neruroblasts (early postmitotic neurons) and anti-MAP-2ab and anti-NeuN label mature neurons. In addition, astrocytes can be stained with anti-GFAP. These markers were used to address the following question in primary postnatal hippocampal cultures: does glucocorticoid treatment target immature or mature neurons for apoptosis? This question deserves attention in view of data showing that dexamethasone (DEX) kills 'hippocampal cells', while at the same time reductions in neurogenesis seen after exposure to stress may be attributed to the elevated levels of corticosteroids experienced during and after a stressful stimulus. In these experiments, analysis was facilitated by including a pharmacological inhibitor of mitosis, arabosinide-C (Ara-C).

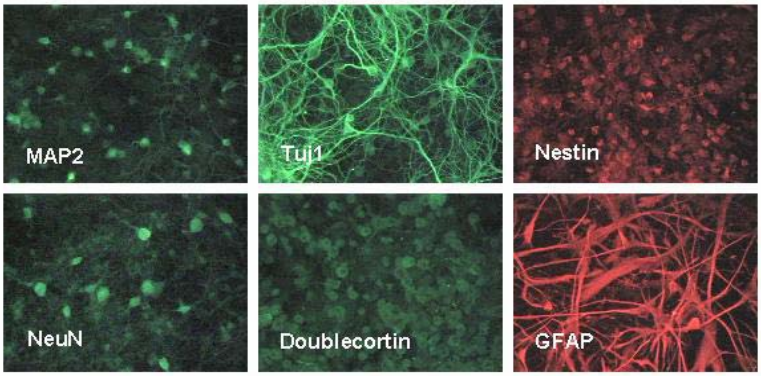



GR antagonists RU38486 and J2700, as well as geldanamycin (gel, an inhibitor of the heat shock protein 90 GR chaperone) attenuate DEX-induced apoptosis in hippocamapl primary cultures.

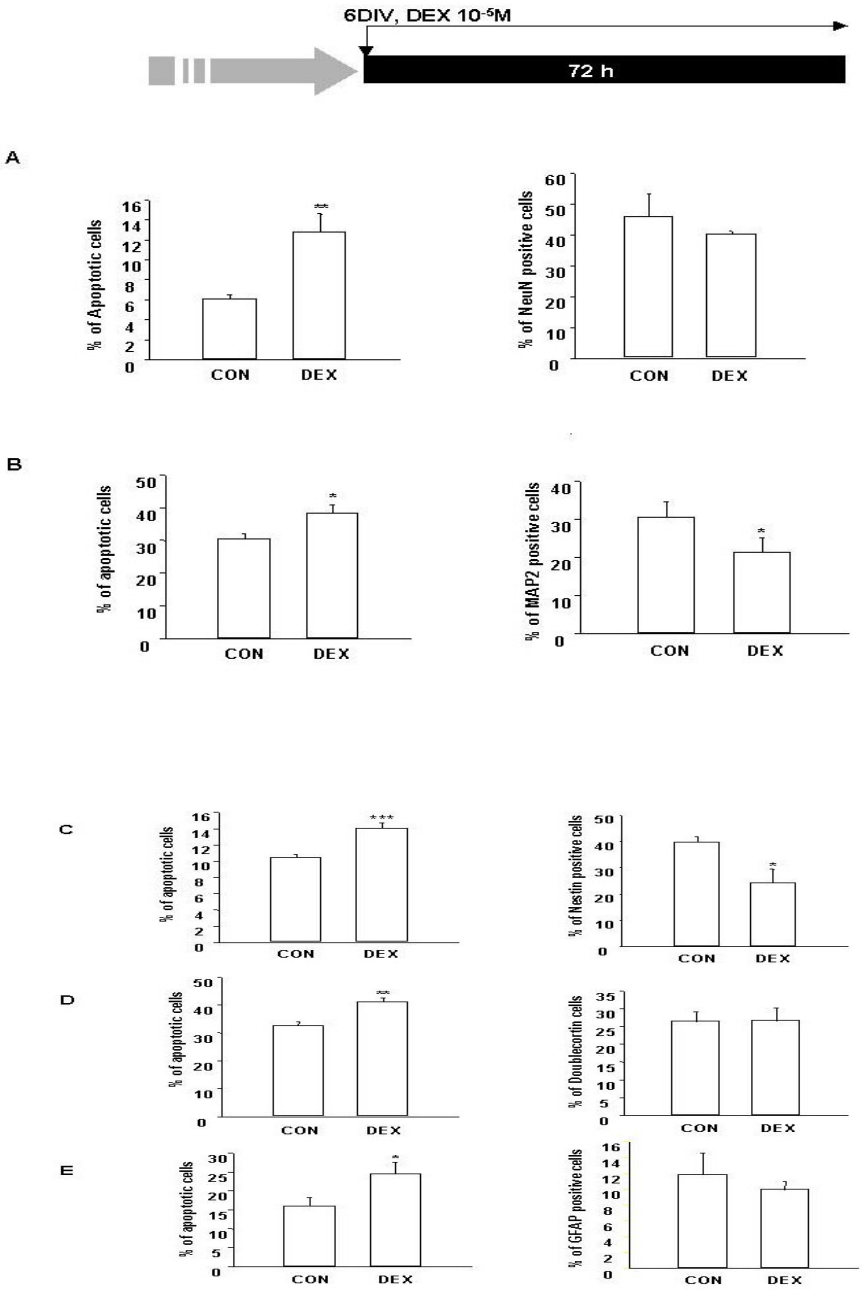
A




B




Semi-quantitative chemo-phenotyping of primary hippocampal cultures using various markers (Nestin for neural precursors, Doublecortin for neuroblasts, TuJ1 for early-mature neurons, MAP2 and NeuN for mature neurons, and GFAP for astroglial cells).




Change in relative numbers of cells displaying various phenotypes after treatment with DEX.

# Chapter 4

## Ionotropic and metabotropic glutamate receptor mediation of glucocorticoid-induced apoptosis in hippocampal cells and the neuroprotective role of synaptic NMDA receptors

4.1. Supplementary background information

4.2. Lu J., Goula D., Sousa N. and Almeida O.F.X. (2003) *Neuroscience*. 121: 123-131

### 4.1. Supplementary background information

Activation of glucocorticoid receptors (GR) leads to neuronal atrophy and death, a phenomenon that has been linked to disorders of mood and cognition as well as neuroendocrine dysregulation (Abraham *et al.*, 2001; Sousa and Almeida, 2002). Our previous studies have clearly demonstrated that glucocorticoid-induced cell death in the hippocampus is apoptotic in nature as judged by morphology and the involvement of specific genetic programmes (Almeida *et al.*, 2000). Several different, although not mutually exclusive, mechanisms have been implicated in these GR-mediated neurodegenerative effects; these include reduced glucose uptake by neurons, increases in the extracellular concentrations of glutamate with concomitant elevations in intracellular $Ca^{2+}$ levels (Sapolsky, 2000).

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4.2. Lu J., Goula D., Sousa N. and Almeida O.F.X. (2003). Ionotropic and metabotropic glutamate receptor mediation of glucocorticoid-induced apoptosis in hippocampal cells and the neuroprotective role of synaptic NMDA receptors. *Neuroscience*. 121, 123-131.

*Neuroscience* 121 (2003) 123–131

# IONOTROPIC AND METABOTROPIC GLUTAMATE RECEPTOR MEDIATION OF GLUCOCORTICOID-INDUCED APOPTOSIS IN HIPPOCAMPAL CELLS AND THE NEUROPROTECTIVE ROLE OF SYNAPTIC *N*-METHYL-D-ASPARTATE RECEPTORS

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**Abstract—Glutamate receptors have been proposed to mediate the apoptotic actions of glucocorticoids in hippocampal cells. To further analyze the role of glutamate receptors in this process, we pretreated primary hippocampal cells from neonatal (postnatal day 4) rats with antagonists of ionotropic glutamate receptor (iGluR) and metabotropic glutamate receptor (mGluR) antagonists before exposure to the specific glucocorticoid receptor agonist dexamethasone (DEX) at a dose of 1 μM. Dizocilpine (MK801; a general *N*-methyl-D-aspartic acid [NMDA] receptor antagonist, NMDAR antagonist) and ifenprodil (a specific ligand of the NMDAR 2B subunit, NR2B), were used to block iGluR; (*RS*)-α-ethyl-4-carboxyphenylglycine (E4CPG) and (*RS*)-α-cyclopropyl-4-phosphonophenyl-glycine (CPPG) were employed as I/II (E4CPG) and II/III (CPPG) mGluR antagonists. Blockade of iGluR resulted in a significant attenuation of DEX-induced cell death; the finding that ifenprodil exerted a similar potency to MK801 demonstrates the involvement of NR2B receptors in glucocorticoid-induced cell death. Apoptosis accounted for a significant amount of the cell loss observed, as detected by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling histochemistry for the *in situ* labeling of DNA breaks; apoptotic cells were distinguished from necrosis on the basis of morphological criteria, including chromatin condensation, membrane blebbing and presence of apoptotic bodies. Treatment with E4CPG and CPPG completely abolished the apoptotic response to DEX, thus showing the additional contribution of mGluR to the phenomenon. Further, dose-response studies with NMDA revealed that whereas high (10 μM) doses of NMDA themselves elicit cytotoxic responses, low (1–5 μM) concentrations of NMDA can effectively oppose DEX-induced cell death. Interestingly, the neuroprotective actions of low dose NMDA stimulation were abolished when either synaptic or extrasynaptic NMDA receptors were blocked with MK801 in combination with the GABA receptor antagonist bicuculline (synaptic) or ifenprodil (extrasynaptic). In summary, the present data show that both iGluR and mGluR mediate the neurotoxic effects of glucocorticoids on hippocampal cells and that pre-treatment with low doses of NMDA, by acting on synaptic and extrasynaptic receptors, render hippocampal cells less vulnerable to glucocorticoid insults.** 

**Key words: glucocorticoids, glutamate, NMDA, ionotropic glutamate receptor, metabotropic glutamate receptor, hippocampus.**

Glucocorticoids (represented endogenously by cortisol and corticosterone) are hormones secreted by the adrenal cortex in response to stress. Acting through hippocampal receptors (glucocorticoid receptors [GR]), they play an important role in the physiological and behavioral adaptive responses to stress (Sapolsky et al., 2000). On the other hand, there is an abundant literature indicating that excessive glucocorticoid secretion can result in damaging effects on adaptive mechanisms as well as on the viability of structural integrity of neurons in the hippocampus. Notably, activation of GR leads to neuritic atrophy and death of neurons, a phenomenon that has been linked to disorders of mood and cognition as well as neuroendocrine dysregulation (De Kloet et al., 1998; McEwen, 2000; Sapolsky, 2000; Abraham et al., 2001; Sousa and Almeida, 2002). Despite some conflicting reports (Masters et al., 1989; Roy and Sapolsky, 2003), work from our laboratory has demonstrated that apoptosis is at least one of the forms of cell death resulting from exposure to glucocorticoids; that conclusion was made in light of the presence of specific morphological features (see Hassan et al., 1996) and the activation of key pro-apoptotic versus anti-apoptotic genes (Almeida et al., 2000). Several different, although not mutually exclusive, mechanisms have been implicated in these GR-mediated neurodegenerative effects; these include reduced glucose uptake by neurons, increases in the extracellular concentrations of glutamate with concomitant elevations in intracellular $Ca^{2+}$ levels (Sapolsky, 2000).

Glutamate is the dominant neurotransmitter in the brain, being found in most excitatory synapses. Its actions are mediated by ionotropic glutamate receptors (iGluR) which are directly associated with ligand-gated ionophores permitting $Ca^{2+}$ influx, and metabotropic glutamate receptors (mGluR); the latter are G protein-coupled receptors which can activate secondary messengers such as cAMP

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*Abbreviations:* CPPG, (*RS*)-α-cyclopropyl-4-phosphonophenylglycine; DEX, dexamethasone; E4CPG, (*RS*)-α-ethyl-4-carboxyphenylglycine; EBBS, Earle's buffered salt solution; GluR, glutamate receptor; GR, glucocorticoid receptor; iGluR, ionotropic glutamate receptor; mGluR, metabotropic glutamate receptor; MK-801, dizocilpine; MR, mineralocorticoid receptor; NMDA, *N*-methyl-D-aspartic acid; NMDAR, NMDA receptor; NR2A, NMDA receptor subunit 2A; NR2B, NMDA receptor subunit 2B; P, postnatal day; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

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or diacylglycerol and phosphoinositides (Lipton and Rosenberg, 1994; Pin and Duvoisin, 1995). Both types of receptor can activate downstream pathways that determine cell survival or death. Their dichotomous actions appear to result from differences in the duration and magnitude of action and the subsequent levels of intracellular $Ca^{2+}$. Synaptic *N*-methyl-D-aspartic acid (NMDA) receptor (NMDAR), which respond to physiological (low dose) NMDAR activation, have been associated with the promotion of cell survival; in contrast, extrasynaptic NMDAR located on the cell body, axon and dendritic spines (Li et al., 1998; Tovar and Westbrook, 1999) have been associated with apoptosis (Hardingham et al., 2002; Riccio and Ginty, 2002). As compared with the synaptic NMDAR, the extrasynaptic NMDAR is characterized by a faster and more extensive rundown of peak current (Li et al., 2002). It is currently believed that while activation of extrasynaptic NMDAR is a rare event under normal conditions, it becomes more common during acute cellular insults or pathological situations when glutamate transporters operate in reverse, thereby increasing extracellular concentrations of glutamate (Rossi et al., 2000).

Gluocorticoids and glutamate receptor (GluR) share an intimate relationship: (i) glucocorticoids can increase hippocampal cell vulnerability to GluR activation (Armanini et al., 1990); (ii) GR activation leads to an up-regulation of the expression of one type of GluR, the ionotropic NMDAR, strongly implicated in neurotoxicity (Weiland et al., 1997); (iii) elevated glucocorticoid levels have been associated with an increase in glutamatergic transmission (Moghaddam et al., 1994) and (iv) glutamate can enhance GR activation (Gursoy et al., 2001). In light of these interactions, and given the neurotoxic potential of each, glutamate and glucocorticoids, the many possibilities for mutual potentiation of each other's effects can be easily appreciated.

Blockade of the NMDAR was previously shown to mediate the inhibitory effects of stress (during which glucocorticoid secretion is increased) on neurogenesis in the hippocampus; this observation provides a clue as to the particular subtype of GluR that may mediate at least some glucocorticoid actions (Gould et al., 1997). The studies described here were designed to provide further insight into the role of GluR in dexamethasone (DEX)-induced cell death in the hippocampus. To do so, we resorted to an *in vitro* model, treating primary hippocampal cells from postnatal day 4 (P4) rats with ionotropic and metabotropic GluR antagonists, to evaluate the role of each of these receptors in DEX-induced apoptosis. A further objective of this study was to explore the possibility of an additive effect of DEX and NMDA; for this, hippocampal cells were exposed to a range of NMDA concentrations before subsequent treatment with DEX. Finally, the role of extrasynaptic versus synaptic NMDAR in these events was examined with the aid of ifenprodil, a selective antagonist of extrasynaptic NMDA-R, and a combination of MK-801 and bicuculline to block synaptic NMDAR.

## EXPERIMENTAL PROCEDURES

### Primary cell culture

Cultures were prepared from Wistar rats aged 4 days (P4; Charles River, Sulzfeld, Germany), following a protocol previously developed in our laboratory (Crochemore, 2000). Briefly, hippocampal slices were digested using the Papain Dissociation System from Worthington Biochemicals (Lakewood, NJ, USA) and the dissociated cells plated on poly-*d*-lysine-coated glass coverslips at a density of 400 cells/$mm^2$. Cultures were maintained in Neurobasal A medium to which 2% B27 supplement, 1 mM GlutamaxI (Invitrogen, Eggenstein, Germany) and 0.1mg/ml kanamycin were added (all supplements from Invitrogen). Culture medium was half-renewed every 3 days. Experiments were started 6 days after plating. Immunocytochemical analysis of the cultures revealed that the cultures comprised ca. 90% neurons (neuronal markers used: NeuN, TuJ1 and doublecortin) and ca. 10% astro-glial cells (glial fibrillary acidic protein-positive).

### Drugs

DEX, obtained as a freely-soluble sodium salt from Merck (Darmstadt, Germany), was used at a concentration of 1 μM in all experiments; DEX treatment was always added to cultures after 3–6 days *in vitro* for 72 h. In all experiments, the mineralocorticoid receptor (MR) antagonist spironolactone was added (10 μM) in order to antagonize interfering effects from low (nanomolar range) MR-activating doses of corticosterone present in the culture medium (Crochemore, 2000). NMDA was obtained from Sigma Chemicals (Deisenhofen, Germany). All other drugs were purchased from Tocris (Bristol, UK). They included bicuculline (used at 50 μM), MK801 (a general NMDAR antagonist; used at 10 μM), ifenprodil (a specific ligand of the NMDAR 2B subunit; used at 10 μM), and (*RS*)-α-ethyl-4-carboxyphenylglycine (E4CPG) and (*RS*)-α-cyclopropyl-4-phosphonophenyl-glycine (CPPG), antagonists of mGluR I/II and mGluRII/III, respectively (both used at 10 μM). The precise treatment protocols used in individual experiments are detailed below.

### Experiment 1: attenuation of DEX-induced apoptosis by GluR blockade

Hippocampal cells were pretreated with either MK801, ifenprodil or a combination of CPPG and E4CPG for 15 min before addition of DEX (continued presence of antagonists) for 72 h after which cultures were analyzed for the incidence of apoptosis.

### Experiment 2: dose-dependent effects of NMDA on DEX-induced cell death

In the first part of this experiment, cells were treated with NMDA for 15 min at doses between 1 and 10 μM; assessment of cell death (apoptosis and necrosis) was performed 72 h later. In order to maximize the NMDA effects, the usual culture medium (supplemented Neurobasal A) was replaced by and added to the cells in $Mg^{2+}$ free Earle's buffered salt solution (EBSS; Invitrogen) containing 10 μM glycine. The treatment schedule for the second part of the experiment was as follows: pre-exposure to NMDA (1–10 μM in $Mg^{2+}$ free EBSS plus 10 μM glycine) for 15 min, followed by a washout step, and addition of DEX (1 μM in supplemented Neurobasal A) for 72 h. At this point, the cultures were fixed and processed for the detection of apoptosis.

### Experiment 3: role of synaptic receptors in NMDA-associated neuroprotection

After pre-exposure to a mixture of bicuculline and MK801 (50 μM and 10 μM, both in $Mg^{2+}$ free EBSS plus 10 μM glycine) for 15 min, a washout step, NMDA (1 or 5 μM) was added and incubation

continued for a further 15 min. A thorough washout step was then performed and cells were subsequently treated with DEX (1 μM in supplemented Neurobasal A) and maintained for 72 h when they were processed for the detection of apoptosis.

### Experiment 4: attenuation of NMDA-induced neuroprotection by ifenprodil

After pre-exposure to ifenprodil (10 μM) and NMDA (1 or 5 μM in $Mg^{2+}$ free EBSS plus 10 μM glycine) for 15 min, and a washout step, cultures were then transferred to supplemented Neurobasal A medium containing DEX at a concentration of 1 μM for 72 h before being examined for apoptosis.

### Cell death assay

Cell death was examined in 4% paraformaldehyde-fixed cells by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) histochemistry or Hoechst 33342 staining. When TUNEL histochemistry was to be performed, permeabilization (0.1% Triton X-100) and peroxidase blocking (1% $H_2O_2$) steps were carried out before application of the TUNEL assay as previously described (Almeida et al., 2000). Apoptotic cells were characterized by dark brown nuclear staining; only those nuclei showing evidence of DNA fragmentation without plasma membrane damage were taken to be apoptotic cells. Hoechst staining was used in a few cases to confirm TUNEL staining. Fixed cells were incubated with the dye (1:1000) for 15 min before examination under a fluorescence microscope. Apoptotic versus total cells (%) were quantified in at least five randomly chosen microscopic fields (0.072 $mm^2$, magnification of 400×) across the long axis of the coverslips on which cells were grown; an average of 1000 cells were sampled on each coverslip and the results shown represent values from six to 10 coverslips per treatment.

### Statistical analysis

All data are depicted as means±S.D. and represent the observations from three to five independent experiments, with three to four replicates for each data point. Data were analyzed for statistical significance using ANOVA and appropriate post hoc tests (Student-Newman-Keuls or Kruskal-Wallis multiple comparison procedures) in which $P \leq 0.05$ was set as the minimum level of significance.

## RESULTS

Throughout, TUNEL-positive cells were considered to be apoptotic only if they showed the characteristic morphological features of apoptosis (see Hassan et al., 1996). Under control conditions (no drug treatment), basal rates of apoptosis were 10.9±1.9% (TUNEL-positive cells meeting morphological criteria of apoptosis expressed as a percentage of total number of cells; mean±S.E.M.). Representative microscopic fields from TUNEL-stained control and DEX-treated cultures are shown in Fig. 1.

### Attenuation of DEX-induced apoptosis by GluR blockade (Fig. 2)

Apoptosis was significantly increased in hippocampal cultures exposed for 72 h to the GR-specific agonist DEX at a dose of $10^{-6}$M ($P<0.05$); the apoptotic effects of DEX were abrogated by the GR antagonist RU 38486 (mifepristone, $10^{-5}$M), indicating mediation by GR (data not shown). Subsequent experiments involved pretreatment of cells with various GluR antagonists (at $10^{-5}$M for 30 min before the introduction of, and during exposure to, DEX at $10^{-6}$M). Concomitant treatment with the general NMDAR antagonist MK801 or the NR2B-specific antagonist ifenprodil rescued cells from DEX-induced apoptosis ($P<0.01$) to levels that were not significantly different from those observed under drug-naive conditions. In the absence of DEX, MK801 and ifenprodil did not exert significant effects on the rate of apoptosis in the cultures (data not shown). Application of the mGluR antagonists E4CPG (mGluR I/II) and CPPG (mGluR II/III), while not exerting any effects on their own (data not shown), abolished the apoptotic actions of DEX ($P<0.001$) and significantly improved basal cell survival ($P<0.05$), i.e. the protective effects afforded by the mGluR antagonists exceeded those provided by NMDAR blockade ($P<0.01$ or $P<0.001$).

The results of this experiment therefore conclusively demonstrate that induction of apoptosis by DEX is mediated by GR and, in turn, by iGluR and mGluR.

### Dose-dependent effects of NMDA on DEX-induced cell death (Fig. 3)

In view of the above findings implicating NMDAR involvement in the apoptotic actions of DEX, we first conducted a dose-response study with NMDA. The NMDA was added for 15 min in $Mg^{2+}$-free medium containing 10 μM glycine. As shown in Fig. 3A, the two lower doses of NMDA (1 and 5 μM) did not influence hippocampal survival, as assessed 72 h after application of the drug. In contrast, the highest dose tested (10 μM) led to a significant increase in cell death ($P<0.05$). Based on this result as well as reports that GR activation can increase the vulnerability of hippocampal cells to glutamatergic toxicity (Armanini et al., 1990), we subsequently examined whether pre-exposure to NMDA can influence the magnitude of DEX-induced cell death; in these experiments, cells were pre-treated with NMDA and then exposed to DEX (in the absence of NMDA) for 72 h. As shown in Fig. 3B, the highest dose of NMDA (10 μM) resulted in cell death but did not potentiate the effects of DEX. On the other hand, and contrary to our predictions, NMDA at one and 5 μM was found to significantly attenuate the apoptotic actions of DEX ($P<0.001$ and $P<0.01$).

These results demonstrate that low doses of NMDA can counteract the cell death-inducing effects of DEX.

### Synaptic receptors mediate the neuroprotective effects of NMDA (Fig. 4)

Recent evidence suggests that extrasynaptic NMDAR may be responsible for triggering excitotoxicity; in contrast, synaptic NMDAR are thought to activate cell survival-promoting signaling cascades (Hardingham et al., 2002). In contrast to NMDA receptor subunit 2A (NR2A) subunits which are mainly found in synaptic NMDAR, NMDA receptor subunit 2B (NR2B) predominate in extrasynaptic NMDAR (Tovar and Westbrook, 1999). Because of this, and the fact that we observed that DEX-induced apoptosis could be attenuated by antagonism of the NR2B subunit with ifenprodil (Fig. 2), it was of inter-

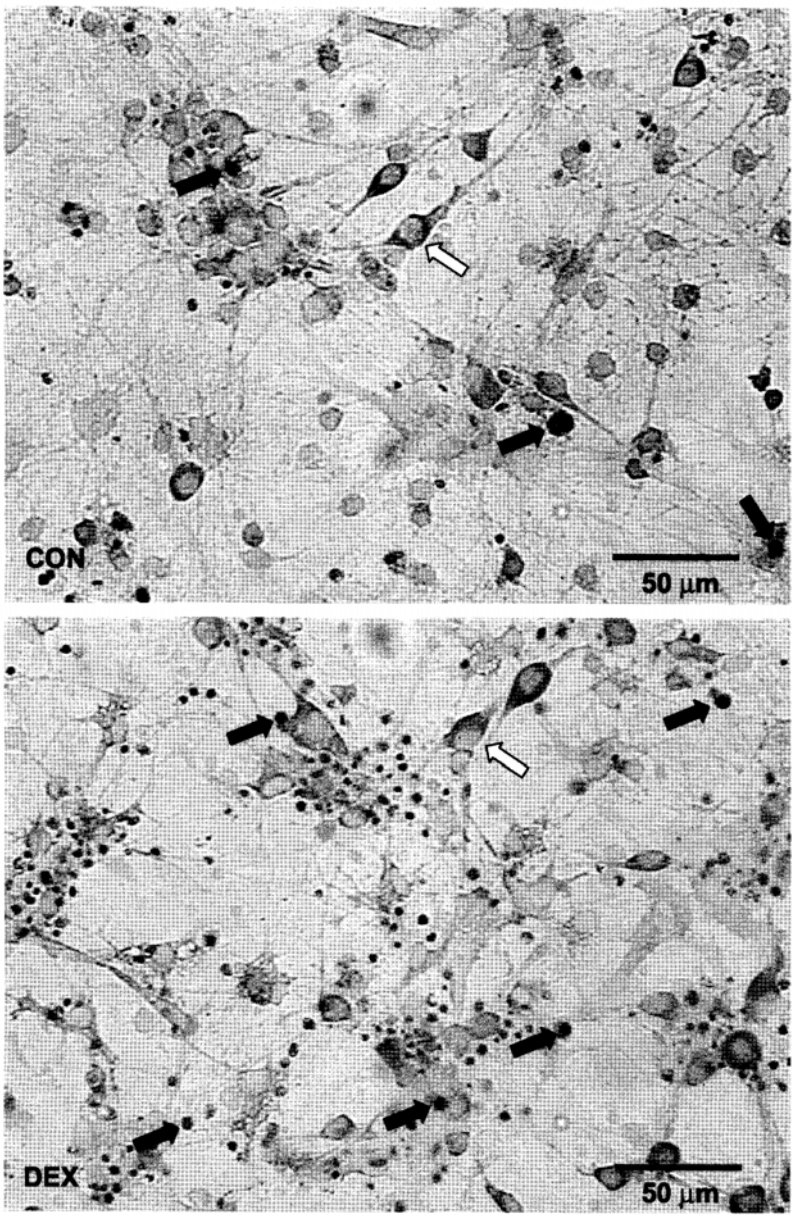


**Fig. 1.** DEX increases apoptosis in primary hippocampal cell cultures. Photomicrographs show TUNEL-staining in control (upper panel) and DEX-treated (lower panel) cells. Examples of TUNEL-positive cells displaying some of the morphological features used to designate apoptotic cells in this study (e.g. shrunken cell body or apoptotic bodies) are marked with black arrows. Note that the relative number of apoptotic versus healthy cells (white arrows) is increased after exposure to DEX (lower panel).

est to examine the role of extrasynaptic NMDAR in our paradigm. Following a recently described experimental paradigm (Hardingham et al., 2002), we applied the GABA receptor antagonist bicuculline (1 μM) together with MK801 (5 μM) in order to subsequently be able to activate extrasynaptic NMDAR selectively. This treatment paradigm abolished the ability of the low doses of NMDA (1 and 5 μM) to counteract the apoptosis-inducing effects of DEX ($P<0.001$ and $P<0.05$).

These findings conform with the view that synaptic NMDAR play a permissive role in the neuroprotective actions of low doses of NMDA.

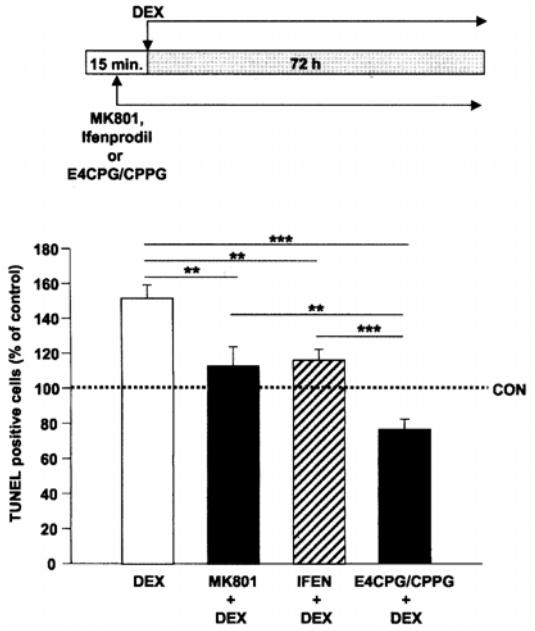


**Fig. 2.** Attenuation of DEX-induced apoptosis by GluR blockade. Treatment of hippocampal cultures with MK801 (NMDAR antagonist), ifenprodil (IFEN; selective NR2B subunit antagonist) and E4CPPG/CPPG (antagonists of mGluRI/II and mGluRII/III, respectively) for 15 min before and during application of DEX (1 μM) resulted in a significant reduction in DEX-induced apoptosis, as measured by TUNEL histochemistry. All of the antagonists were used at 10 μM. ** and *** indicate $P<0.01$ and $<0.001$, respectively. Results represent means±S.D. from five experiments, with three replicates for each data point.

### Ifenprodil attenuates low dose NMDA-induced neuroprotection (Fig. 5)

NR2B are predominantly expressed at extrasynaptic sites which have been associated with neurotoxicity (Hardingham et al., 2002). As mentioned, ifenprodil is a selective inhibitor of the NR2B subunit of the NMDAR (Gotti et al., 1988) and has proven neuroprotective efficacy in animal models of degeneration (Kemp and McKernan, 2002). The results depicted in Fig. 2 show that ifenprodil can reduce the cytotoxic effects of DEX. Since the results of the previous experiment (Fig. 4) indicated that synaptic receptors mediate the protective effects of low doses of NMDA, the question arose as to the impact of NR2B extrasynaptic receptor antagonism on DEX-induced cell death. Because ifenprodil is an activity-dependent NR2B antagonist, cells were pre-treated with NMDA (1 and 5 μM) and ifenprodil (10 μM) for 15 min, before wash-out and exposure to DEX (1 μM) for 72 h. Contrary to our prediction, hippocampal cultures treated according to this paradigm displayed more apoptosis than those exposed only to the NMDA pulse followed by DEX ($P<0.01$ and $P<0.05$). Thus, these observations indicate that transient blockade of NR2B can prevent low doses of NMDA from exerting protective actions against DEX-induced apoptosis.

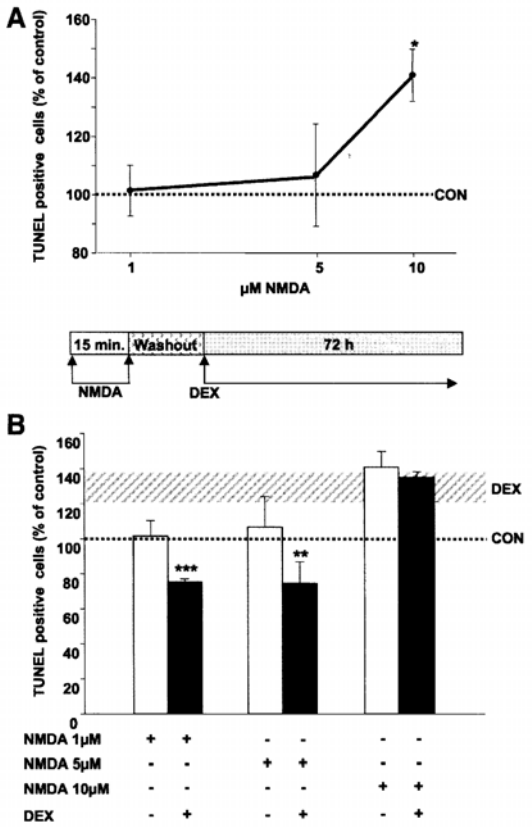


**Fig. 3.** Dose-dependent effects of NMDA on DEX-induced cell death. Cell death was assessed after treatment of cultures for 15 min with NMDA ranging from 1 to 10 μM. A. Dose-response curve showing that 1 and 5 μM NMDA did not have affect on cell survival, whereas 10 μM of the drug significantly stimulated cell death. Note that data shown refer only to counts of TUNEL-positive cells, although after exposure to 10 μM NMDA, there was an increase in the number of cells showing signs of necrosis. B. Demonstration that pretreatment with low, subtoxic doses of NMDA (1 and 5 μM) can attenuate DEX-induced apoptosis. The stippled area represents the effects of treatment with 1 μM of DEX for 72 h. All values shown are means±S.D., derived from five experiments (in which each data point was replicated four times). *, ** and *** indicate significant differences where $P<0.05$, $<0.01$ and $<0.001$, respectively.

## DISCUSSION

Glucocorticoids fulfill several important functions in the hippocampus, including cognition, mood and neuroendocrine regulation. Nevertheless, high levels of both endogenous and therapeutic glucocorticoids can affect hippocampal function deleteriously. These undesired effects have been proposed to result from either neuronal atrophy and, in extreme cases, neuronal cell death of certain principal cells of the hippocampus (for review, see Sousa and Almeida, 2002).

Glucocorticoids can exert rapid, transient effects on neuronal excitability involving increased cytosolic concentrations of $Ca^{2+}$ (Nair et al., 1998). However, glucocorticoid actions are best known to be mediated through GR which are transcription factors and our group has previ-

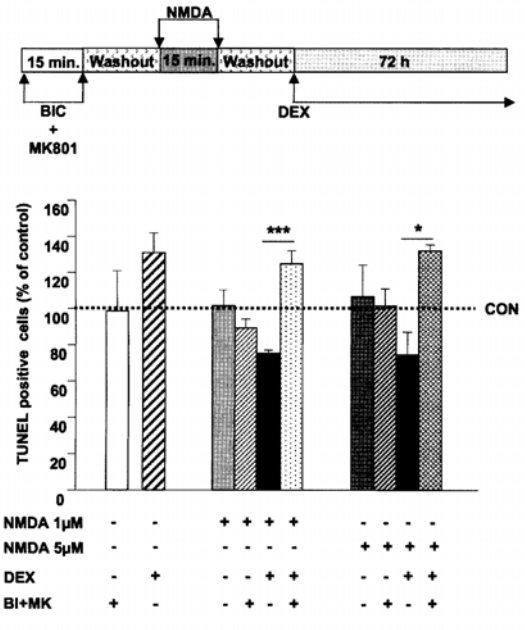


**Fig. 4.** Synaptic receptors mediate the neuroprotective effects of NMDA. Synaptic NMDAR were blocked by initially exposing hippocampal cultures to the GABA antagonist bicuculline (50 μM) and the activity-dependent NMDAR antagonist MK801 (10 μM). Subsequently, cells were treated with NMDA (1 or 5 μM; 15 min), washed and exposed to DEX (1 μM) for a further 72 h when apoptosis was measured. Results (means±S.D. from four experiments, with four replicates for each data point) show that blockade of synaptic NMDAR prevents low doses of NMDA to counteract the apoptotic actions of DEX. * and *** indicate significant differences where $P<0.05$ and 0.001, respectively.

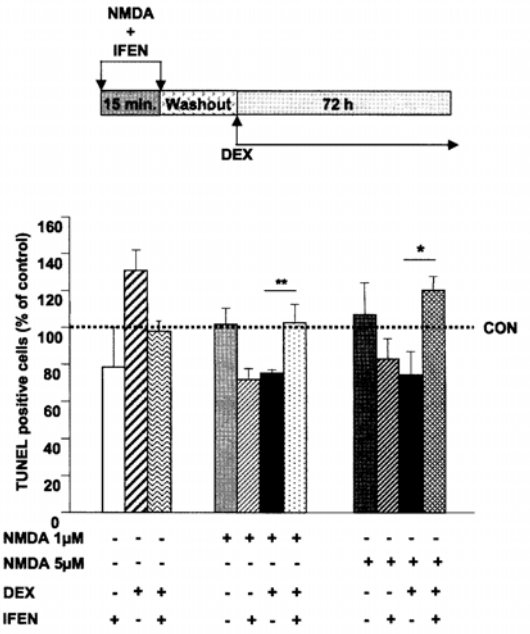


**Fig. 5.** Ifenprodil attenuates low dose NMDA-induced neuroprotection. Pharmacological blockade of the NR2B subunit of the NMDAR was achieved by pre-incubating cells with ifenprodil (IFEN, 10 μM). Subsequently, the ability of low doses of NMDA (1 or 5 μM) to protect against the apoptotic effects of DEX (1 μM; 72 h) was examined. The data (means±S.D. derived from three experiments, with three replicates for each data point) show that the protective effects of NMDA are markedly attenuated when NR2B-containing NMDAR are rendered non-functional by IFEN. * and ** indicate significant differences where $P<0.05$ and $<0.01$, respectively.

ously elucidated some of the cell death-related molecular pathways triggered by exposure to the potent GR agonist DEX (Almeida et al., 2000). Thus, glucocorticoid effects on hippocampal structure and function do not necessarily result from one exclusive mechanism (electrophysiological versus genomic) but rather may occur as a result of integrated signals arising from both the cell membrane and transcriptional activity. For example, by repressing the transcription of $Ca^{2+}$ channel and $Ca^{2+}$ extrusion pump genes (Bhargava et al., 2000), glucocorticoids contribute to long-term alterations in the dynamics of intracellular $Ca^{2+}$ levels, including those originating at the plasma membrane (Kerr et al., 1992; Elliott and Sapolsky, 1993; Nair et al., 1998; Bhargava et al., 2000).

Exaggerated and chronic elevations in intracellular $Ca^{2+}$ accompany glutamatergic excitotoxicity (Choi, 1991; Coyle and Puttfarcken, 1993) and have also been proposed to at least partially underlie the neurotoxic effects of glucocorticoids (Joëls, 2001). Increases in cytosolic $Ca^{2+}$ concentrations result from the activation of both iGluR and mGluR, albeit through different mechanisms: iGluR stimulate the influx of $Ca^{2+}$ from the extracellular space, whereas mGluR mobilize $Ca^{2+}$ from intracellular reservoirs (Maiese et al., 1999; Otani et al., 2002). Glucocorticoids are known to increase NMDAR expression and glutamate synthesis and extracellular accumulation (Weiland et al., 1997; Moghaddam et al., 1994), and to potentiate glutamate-induced cell death (Goodman et al., 1996; Behl et al., 1997; Abraham et al., 2001; Johnson et al., 2002). To date, however, earlier suggestions that glutamatergic mechanisms may play an intermediary role in glucocorticoid-induced cell death have remained largely unsupported by firm experimental evidence. The data presented in this paper fill that gap by demonstrating that iGluR (here, only the NMDAR type was studied) and mGluR mediate at least some of the apoptotic effects of DEX in primary hippocampal cultures. With respect to the involvement of iGluR, our data show that general blockade of NMDAR with MK801 significantly attenuates DEX-triggered cell death (Fig. 2).

GluR are well-recognized triggers of neuronal cell death and may occur either acutely upon activation or after a period of delay (Choi, 1991). These two forms of neuronal death are distinguishable on the basis of their morphological characteristics and ionic dependence. The rapid-onset form is necrotic in nature, characterized by cell swelling, intact nuclei with diffuse nucleoplasm, disrupted cell membrane and ultimate cell lysis. In contrast, the delayed

form is $Ca^{2+}$-dependent, and is accompanied by cell shrinkage, membrane blebbing and nuclear condensation and fragmentation (karyoklasis); all of the latter features are typical of apoptosis (Choi, 1991). In the present work, the dominant form of cell death observed following treatment of hippocampal cells with both NMDA and DEX was of the apoptotic type.

The NMDAR has been the most intensely studied GluR, especially in the context of neurotoxicity. NMDAR are heteromeric in nature; they consist of a common NMDA receptor subunit 1 and one or more NR2 subunits (NR2A-D), whose insertion in the NMDAR complex varies during development and maturity of synapses (Li et al., 1998; Stocca and Vicini, 1998; Tovar and Westbrook, 1999). For example, it is known that whereas NR2A is predominantly found in NMDAR located at the synapse, NR2B are localized almost exclusively in NMDAR at extrasynaptic (non-synaptic) sites. An important concept that has emerged from recent studies is that synaptic NMDAR can initiate neuroprotective mechanisms; in contrast, activation of extrasynaptic NMDAR results in cell death (Hardingham et al., 2002). The present experiments demonstrate that selective inhibition of NR2B with ifenprodil results in a suppression of DEX-induced apoptosis (Fig. 2); this finding is consistent with the documented efficacy of ifenprodil in retarding cell death in various animal models of neurodegenerative disease (for review see Chenard and Menniti, 1999).

Previous work which showed that GluR stimulation can result in excitotoxicity and that the latter can be amplified by pre-treatment with glucocorticoids (Goodman et al., 1996; Behl et al., 1997; Abraham et al., 2001; Johnson et al., 2002) prompted us to examine the effects of NMDA pre-treatment on DEX-induced apoptosis. To do this, we treated hippocampal cultures with NMDA at doses ranging from 1 to 10 μM for a brief period (15 min) before exposure to DEX (1 μM) for 72 h. We observed a complete abolition of the apoptotic actions of DEX when cells were pre-exposed to NDMA at 1 and 5 μM; at a dose of 10 μM, NMDA resulted in overt necrosis (Fig. 3). Subtoxic concentrations of NMDA are indeed known to elicit neurotrophic and anti-apoptotic mechanisms in neurons (Marini et al., 1998; Resink et al., 1996; Brandoli et al., 1998); moreover, one of the implicated neurotrophins, brain-derived nerve growth factor, has been found to stimulate (pro-survival) NR2A subunit expression and to suppress NR2B (death-promoting) expression (Glazner and Mattson, 2000). Because our experimental paradigm involved chronic exposure to DEX, it is pertinent to mention a study which showed that GR activation leads to an increase in NR2B subunit gene expression with a concomitant decrease in the expression of the gene encoding NR2A subunit (Nair et al., 1998).

As noted above, NMDAR including the death-promoting NR2B subunit are predominantly localized at extrasynaptic sites; in contrast those comprising the pro-survival NR2A subunit have a synaptic location (Riccio and Ginty, 2002). Since the appearance of NR2A- and NR2B-containing NMDAR is correlated with the ontogeny of synapses (Tovar and Westbrook, 1999; Li et al., 1998; Stocca and Vicini, 1998), it is pertinent to note that although the cultures used for the present studies were relatively young (experiments carried out after 6 days *in vitro*), cell–cell interactions were abundantly evident at the light microscopic level. In order to distinguish between synaptic and extrasynaptic NMDAR in the mediation of the protective actions of low doses of NMDA against DEX-stimulated apoptosis, we adopted the recently described elegant pharmacological paradigm described by Hardingham et al. (2002). Briefly, the paradigm which consists of pre-treating (15 min) hippocampal cultures with the GABA antagonist bicuculline (to activate synaptic GluR) and MK801 (to block active NMDAR) prevents NMDA activation of neuroprotective signaling cascades, leaving only extrasynaptic NR2B-containing NMDAR available for NMDA binding. Cultures pre-treated in this way were subsequently exposed to 1 or 5 μM NMDA and DEX, after which they were analyzed for apoptosis. The observation that the bicuculline/MK801 pre-treatment abrogated the ability of NMDA to oppose the apoptotic actions of DEX (Fig. 4) is consistent with a synaptic site of NMDA-induced neuroprotection (cf. Hardingham et al., 2002).

In an experiment to examine the impact of NR2B subunit-containing NMDAR blockade on the neuroprotection afforded by subtoxic NMDA doses against DEX-elicited apoptosis, NR2B blockade was achieved by pre-treating cells with NMDA and ifenprodil. It was reasoned that this experimental design would ensure binding of ifenprodil, an activity-dependent antagonist, to extrasynaptic receptors and, at the same time, permit (or even enhance) the activity of synaptic NMDAR. Contrary to expectations, we observed that the NR2B-blocking procedure led to an amplification of DEX-induced apoptosis (Fig. 5). Only speculative explanations can be offered for this finding at present. Assuming similar affinities of NMDA for NR2A and NR2B, and that neuroprotective NR2A are predominantly occupied under basal conditions, a tenable explanation is that, as extracellular glutamate concentrations increase, neuronal fate is balanced by NR2A versus NR2B activity. Also, since the NMDA/ifenprodil pre-treatment was transient (15 min), it could be that the prolonged exposure to DEX resulted in increased sensitivity and/or up-regulation of the synthesis of death-promoting NR2B receptors, or equally, a down-regulation of pro-survival NR2A receptors; the likelihood of such a mechanism is supported by the observation that extended exposure to ifenprodil abrogated the neurotoxic actions of DEX (Fig. 2). In this context, it should be noted that the GR is a potent transcriptional factor. Thus, another plausible explanation would be that by employing transcriptional mechanisms to elevate glutamate synthesis (Ábrahám et al., 1996), DEX would effectively make more neurotoxic glutamate available for activating NR2 subunit NMDAR, thus positively driving a vicious circle. Further studies are required to clarify these issues.

A role for mGluR was demonstrated by the finding that E4CPG and CPPG, selective antagonists of mGluR I/II and II/III, respectively, can rescue hippocampal cells from

DEX-induced apoptosis; strikingly, it was observed that mGluR antagonism can also improve neuronal survival under basal conditions (Fig. 2). In general, the manifestation of mGluR-mediated actions are slow and involve gene activation. Type I mGluR have been associated with neuronal death (Snyder et al., 2001; Allen et al., 2001; Miskevich et al., 2002; Heidinger et al., 2002) while Type II/III mGluR have been shown to contribute to neuronal survival. The present results, using a cocktail of relatively non-selective antagonists because of the unavailability of more receptor type-specific drugs are interesting: they suggest the involvement of either complex regulatory interactions between the various mGluR or the up-regulation of neurotoxic type I mGluR by DEX.

The present paper represents the first attempt to understand the mechanistic and functional nature of interactions between GluR and glucocorticoids. Its results demonstrate that the apoptotic actions of DEX are at least partly mediated by GluR of the NMDA and metabotropic types. In addition, the results reported herein show that low doses of NMDA, acting via synaptic NMDAR can effectively block hippocampal cell death induced by DEX. Last, this work indicates that glucocorticoids can cause apoptosis in hippocampal cells by triggering rapid (NMDAR-mediated) as well as slow (mGluR- or GR-mediated) responses, and that the final outcome of glucocorticoid treatment on hippocampal cell survival depends on the convergence and integration of transcriptional signals (e.g. GluR and agonist availability; regulation of apoptosis-related genes) and signals originating at the cell membrane (e.g. $Ca^{2+}$ conductance). Although the present findings are consistent with previous findings, it should be noted that the present experiments were carried out on cells obtained from neonatal hippocampal tissue; therefore, the mechanisms described here do not necessarily apply to the adult hippocampus which has been the focus of the majority of *in vivo* studies of the neurotoxic actions of glucocorticoids. A second caveat concerns the fact that the serum-free medium used for the cell cultures favored the survival of neurons rather than glia (neuronal–glial ratio approximately 10:1 versus 1:10 *in vivo*). Since astrocytic glutamate transporters are important for maintaining low extracellular glutamate concentrations (Nedergaard et al., 2002), the results obtained here more likely represent an extreme situation rather the physiological norm. In this context, it is also important to remember that the dose of DEX (1 μM) used in these *in vitro* experiments is high in relation to the nanomolar concentrations of bioavailable corticosterone that brain neurons are exposed to (Linthorst et al., 2000). Therefore, caution is required in directly extrapolating the present observations to the situation in the organism.

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# Chapter 5

## General discussion

## General Discussion

A central problem in developmental neurobiology concerns *how the balance between neuronal birth, differentiation and death are maintained in the CNS, what factors and regulatory mechanisms might be involved in these processes, and how neuronal development is co-ordinated spatially and temporally*. These questions formed the basis of the work reported in this thesis.

Primary hippocampal granule cell cultures were used to mimic the early postnatal period when the transition from high proliferation to differentiation occurs (i.e. when the subgranular layer [SGZ] begins to appear); this period represents a critical developmental window when pro-neuronal factors increase and pro-proliferation factors decrease, as well as a time when program cell death is prominent. Also used were slightly older cerebellar granule cells cultures originating from animals in which the external granular layer (EGL) had begun to disappear. Together, these model systems served to investigate the regulation of proliferation and neuronal differentiation by secreted growth factors, glucocorticoids and NMDA. Attempts were also made to understand the underlying molecular mechanisms of these processes. Although not directly addressed in the main work presented here, the importance of spatial arrangements is illustrated in the results described in Appendix 1 based on an *in vivo* study on the rat hippocampus.

### 5.1. Experimental approaches

In order to facilitate analysis of the factors and mechanisms regulating neuronal development, experiments were carried out using *in vitro* models: primary dissociated hippocampal and cerebellar cell cultures, hippocampal and cerebellar slice cultures, and a neural cell line (HiB5) derived from hippocampal progenitor cells. Depending on the specific question being addressed, the primary cell and slice cultures were obtained from rats of different postnatal developmental stages. While more amenable to genetic manipulations and pharmacological treatments, and providing for convenient replication of experiments in a given set of neural phenotypes under controlled environmental conditions, these model systems have a number of obvious limitations however. Primary concerns associated with such systems is the lack of the neural matrix found *in vivo*, and the high likelihood that the intercellular connections seen do not faithfully represent those found *in situ*; the latter problem may be accentuated by the fact that mature neurons are less likely to survive the dispersal and cultivation conditions than immature ones. Further, at least with respect to the dissociated cell cultures, between-experiment reproducibility can be easily compromised by variations in the starting quality and cellular composition (e.g. relative numbers of neurons to glia, progenitor versus mature neurons) of the cultures. In light of these reservations, it is essential that extrapolations from the situation *in vitro* to that *in vivo* be made with extreme caution.

Immunohistochemical detection of cells incorporating the thymidine analogue, bromodeoxyuridine (BrdU), served to gain an insight of cell proliferation in the studies described here. This widely used method is more convenient than the traditional one involving the labelling of cells with $^{3}$H-thymidine, but is associated with the risk of detecting cells entering apoptosis unless it is used with extreme caution (e.g. discarding BrdU-labeled cells showing nuclear condensation and fragmentation and membrane blebbing). In some instances, mitosis was confirmed with the aid of Hoechst 33342 dye which exclusively labels DNA, allowing the identification of cell nuclei.

Neurons, in various stages of maturity were identified immunocytochemically with the aid of specific antisera. Immature neurons (neuroblasts) were marked with anti-doublecortin, anti-TuJ1, anti-MASH and anti-Math1, whereas mature neurons were marked with anti-MAP2a,b. The majority of non-neuronal cells in cultures showed the morphological characteristics of astro-glial cells, and were labelled with anti-GFAP.

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) was used to detect apoptotic cells. Appendix 2 provides a detailed critique of the various methods available to detect apoptosis; despite several reservations, TUNEL still remains the most reliable and commonly-used method for detection of apoptosis.

Appendix 2 also includes a discussion about methods for the quantification of cell numbers in a given physiological state, using stereological tools. The latter, which are easily applied to histological sections were not used in the cell-based studies described in the main work described here. Rather, we resorted to a variation of the Abercrombie counting method, and data are usually presented with respect to total number of cells in several randomly-chosen, but representative, microscopical fields.

Both pharmacological and gene transfection approaches were applied to identify some of the mechanisms controlling neuronal birth, maturation and death. Transfections were done using a non-viral approach, mainly with the help of Lipofectamine 2000™ and, in some cases, the cationic polyethylenimine (PEI). The precise conditions and ratio of DNA:transfectant was carefully determined for primary neurons, assuring good survival of cultures and with a reasonable rate of transfection (ca. 5%) which provided reliably quantifiable results (counts of green fluorescent protein (GFP)-labeled cells, translocation of GFP-fused proteins from the cytoplasm to nucleus, and reporter gene assays).

### 5.2. Endocrine and paracrine factors in neural development

As described in the General Introduction (Chapter 1), neural development is regulated by temporally and spatially differentially distributed factors, including intrinsic factors like bHLH transcription factors (eg. Mash1, Math1, NgN), cell cycle regulators (e.g. p21, p27, p57) and extrinsic factors like neurotransmitters such as glutamate, cytokines (e.g. TGFβ, BMP), neurotrophins (e.g. BDNF, NGF), and hormones (e.g. glucocorticoids). The neurotransmitters, cytokines and neurotrphins mainly function by autocrine or paracrine mechanisms via membrane receptors, whereas glucocorticoid action are mediated by nuclear receptors; all of these extrinsic signals can trigger a cascade of responses resulting in the induction of proliferation, differentiation and apoptosis (or survival) by the intrinsic factors. The pro-neural proteins (eg. Mash1, Ngn2) can promote neuronal differentiation by downregulating the cell cycle by activating expression of cdk inhibitors (e.g. p27, p21, p57) or by upregulating the expression of neuronal differentiation genes (e.g. NeuroD) and inhibiting gliogenic factors (e.g. LIF, CNTF, BMP) (Bertrand *et al.*, 2002). For example, p27 promotes gliogenesis in the absence of co-expressed bHLH proteins (Ohnuma *et al.*, 2002) and neurotrophins facilitate neuronal differentiation by inducing the expression of Mash1 and Math1 (Ito *et al.*, 2003); BMP2/4 and epidermal growth factor (EGF) were shown to decrease expression of the pro-neural molecule Mash1 (Ahmad *et al.*, 1998; Shou *et al.*, 1999); the mGlu5 receptor is present in zones of active neurogenesis (e.g. such as the cerebellar EGL and hippocampal SGZ) – mGlu5.R expression is highest during the first postnatal week (Di Giorgi Gerevini *et al.*, 2004), correlating with high levels of neurogenesis and glutamate release and probably involving calcium activation of cell cycle inhibitors (Benitez-Diaz *et al.*, 2003). Taken together, neural development is highly programmed with highly temporally and spatially coordinated interactions

between intrinsic and extrinsic factors - increased production of specific neurotrophins or neurotransmitters appear to increase pro-neural transcription factors and cell cycle inhibitors which, in turn, enhance other pro-neural factors and decrease pro-proliferation factors.

Although many studies have addressed the above-mentioned neuronal development pathways, little is known about the interactions between neurotrophins, cytokines, neurotransmitters and hormones in the regulation of proliferation, differentiation, survival and death. The many similarities between hippocampal and cerebellar granule cells raise interesting questions about the timing of the disappearance of the EGL and appearance of the neurogenic SGZ; are these areas controlled by common intrinsic and extrinsic factors and, if so, what is their identity, and what mechanisms do they exploit? This study focussed on candidate extrinsic factors, in particular glucocorticoids, NMDA, BDNF and transforming growth factor β2 (TGFβ2).

Interactions between glucocorticoid and several growth factors have been previously described. For example, glucocorticoid treatment increases NGF, NT-3, trkA, trkB, trkC and bFGF mRNA expression (Roskoden *et al.*, 2004), although the same treatment decreases proliferation of hippocampal and cerebellar granule cells and promotes premature cessation of precursor cell formation in the EGL (see Table 3 in General Introduction). In contrast, adrenalectomy decreases NGF, BDNF and NT-3 mRNA expression (Barbany and Persson, 1992), and enhances cell proliferation and delays disappearance of EGL in cerebellum while promoting hippocampal granule cell birth (Table 3 in General Introduction). One open question with respect to these studies is whether glucocorticoids exert their anti-proliferative actions by activating pro-neural factors or by direct influences on regulators of the cell cycle.

Glucocorticoid actions are mediated by two nuclear receptors, mineralocorticoid (MR) and glucocorticoid (GR) receptors. Whereas activation of MR or blockade of GR promotes cell survival, activation of GR or antagonism of MR leads to hippocampal granule cell death. As already described in the General Introduction (Chapter 1), GR activation upregulates the expression of NMDA.R, which are strongly implicated in neurotoxicity (Weiland *et al.*, 1997). At the same time, GR occupation is associated with increased glutamatergic transmission (Moghaddam *et al.*, 1994) and glutamate facilitates GR activation (Gursoy *et al.*, 2001). These observations indicate intricate interactions exist between glucocorticoids and glutamate receptors; however, little is known regarding the mechanisms of these interactions. Important questions which arise include: can glucocorticoids potentiate NMDA-induced cell death? Do glucocorticoids induce hippocampal cell death through the mediation of glutamate receptors? Also, which cells are targeted for death by glucocorticoids – immature or mature cells? Answers to these and the previously-raised questions were sought during the course of this work using dissociated hippocampal and cerebellar cell culture models. Cells from 4-7 day old rats were cultured for 7-14 days in vitro, thus covering time windows when the hippocampal SGZ was just beginning to develop and when the cerebellar EGL was beginning to disappear. Consistent with the previously-described different developmental profiles of hippocampal and cerebellar granule neurons, cerebellar cultures contained a greater percentage of mature cells, as judged by the expression of MAP2ab.

A. <u>Cytokine regulation of cell birth and differentiation</u>

To study whether secreted growth factors that might contribute to the temporally distinct patterns of hippocampal and cerebellar granule cell development, conditioned medium (CM) exchanges between hippocampal and cerebellar cultures were used in the experiments described in Chapter 2. Interestingly,

$CM_{Hippocampus}$ stimulated proliferation in cerebellar cultures whereas $CM_{Cerebellum}$ treatment inhibited cell proliferation and accelerated neuronal maturation in hippocampal cultures. The latter events were accompanied by increased expression of two cell cycle arrest-related molecules, p21 and p27. Results of subsequent antibody neutralization experiments indicated that the anti-proliferative actions of $CM_{Cerebellum}$ may be due to the presence of BDNF, TGFβ2 and BMP2; involvement of these molecules was confirmed by molecular pathway analysis (described in the next section). Ongoing experiments are using slice-slice and slice-cell co-cultures to obtain further evidence for this. In addition, a newly-developed slice-membrane blotting technique is being set up to investigate whether BDNF and TGF-β2 are found within or in the close vicinity of cerebellar and hippocampal granule cell layers; such evidence would strongly suggest that these factors act in a paracrine or even autocrine mode.

Neurotrophins such as BDNF and NGF exert their actions on neuronal survival and differentiation by signaling through TrK receptors (Hofer *et al*., 1990; Minichiello and Klein, 1996; Borghesani *et al*., 2002; Bandtlow *et al*.,1990; von Bartheld, 1991; Rocamora *et al*., 1993; Segal *et al.*,1995; Zhou and Rush, 1994) which are linked to the activation of Ras-Raf-MAPK pathways. In general, neurotrophins stimulate rather than inhibit proliferation (see Table 1 in General Introduction) and promote neuronal differentiation (Benraiss *et al.*, 2001; Chmielnicki and Goldman, 2002); here, treatment of immature cells with BDNF was found to inhibit proliferation (Chapter 2), and effect that might be related to the observation that BDNF induces p21 expression in maturing cerebellar granule cells (Lin *et al.*, 1998).

There are three isoforms of TGF-β, each derived from separate genes: TGF-β1 whose expression is normally restricted to the choroid plexus, and TGF-β2 and TGF-β3 which are expressed in neurons and glia (Unsicker *et al.*, 1991; Pratt and McPherson, 1997). TGF-β1 and TGF-β3 have been implicated in neuroprotection, while neurotrophic functions have been ascribed to TGF-β2 and TGF-β3 (Finch *et al.*, 1993; Böttner *et al.*, 2000; Pratt and McPherson, 1997). The latter include stimulation (Mahanthappa and Schwarting, 1993) or inhibition (Constam *et al.*, 1994) of neurogenesis, or both (Kane *et al.*, 1996), as well as the regulation of neuronal differentiation (Ishihara *et al.*, 1994; Abe *et al.*, 1996; Cameron *et al.*, 1998). TGF-β2, the isoform focused on in this work, is expressed in the EGL (neurogenic) and in Purkinje and radial glia of the cerebellum according to a strict temporal pattern but interestingly, appreciable levels of TGF-β2 are not seen in other brain sites of neuronal proliferation (Flanders *et al.*, 1991; Constam *et al.*, 1994; Unsicker and Strelau, 2000). The previously-described expression profiles held true in our cultures: TGF-β2 was much stronger in cerebellar vs. hippocampal cells (Chapter 2). Ongoing immunostaining to examine the dynamics of BDNF and TGFβ2 expression in the cerebellum and hippocampus of postnatal rats suggests that the temporal profiles of expression of these two peptides place them in a good position to block proliferation and induce maturity in granule cells from both the cerebellum and hippocampus. Although not tissue-specific these molecules reflect the temporal co-ordination of ligand availability and signaling cascades in two cell types each displaying its unique developmental time-table.

The studies described in Chapter 2 demonstrated important roles for BDNF and TGF-β2 in the maturation of cerebellar and hippocampal granule cells; pilot studies (not presented) have also implicated bone morphogenetic protein-2 (BMP2). While ontogenetic maps and mechanisms of action (see next section) are available for each of these factors, the question arises as to why several factors apparently share in the same function? Is it likely that each extrinsic factor is specifically responsible for the maturation of definitive

neuronal phenotypes that could not be identified by our cell characterization tools? Do all these factors belong to an evolutionarily conserved pool where individual factors can compensate for each other, or are there cross-talk between these factors directly or at the level of signal transduction? This last point is addressed later in this Discussion.

B. Glucocorticoid receptors: maintaining the balance between hippocampal survival and death

The results described in Chapter 3 deal with the role of MR and GR in maintaining the balance between cell survival death through apoptosis. A series of *in vivo* studies in rats previously demonstrated that the GR agonist dexamethasone (DEX) stimulates cell death in the hippocampus as well as in other selected brain regions such as the striatum and substania nigra (Hassan *et al.*, 1996; Almeida *et al.*, 2000; Haynes *et al.*, 2001). However, it remains unclear as to whether these effects occur directly or whether gluocorticoids merely exacerbate the neurotoxic effects of other more potent insults such as excitatory amino acids (Elliott *et al.*, 1993) and reactive oxygen species (Behl *et al.*, 1997). Direct neural actions of DEX have also been questioned on the basis of data showing that DEX has limited access to the brain (de Kloet *et al.*, 1975; Schinkel *et al.*, 1995; Meijer *et al.*, 1998). In addition, observations that adrenalectomy (ADX) leads to apoptosis in the hippocampus (Sloviter *et al.* 1993; Sousa *et al.*, 1997), together with the fact that, by suppressing adrenocortical secretion, DEX produces 'chemical adrenalectomy' boost the argument against direct effects of DEX on hippocampal cell survival. It should be noted however, that the mechanisms underlying ADX- and DEX-induced apoptosis are likely to be distinct since whereas significant levels of apoptosis can be observed within 24 hours of a single injection of DEX (Hassan *et al.*, 1996), the effects of adrenalectomy display different temporal and spatial dynamics (Jaarsma *et al.*, 1992; Hu *et al.*, 1997; Sousa *et al.* 1997). Further, the apoptotic effects of DEX were shown to be blocked with a GR antagonist (Haynes *et al.*, 2001). As mentioned earlier, GR are nuclear receptors which act as potent transcription factors; previous work from this laboratory showed that DEX can trigger a molecular death cascade in both the hippocampus (Almeida *et al.*, 2000) and a neural cell line (Crochemore *et al.*, 2002).

To address the specific question of whether DEX treatment directly causes apoptosis in the hippocampus, we here used primary hippocampal cultures which we demonstrated to express both MR and GR. The experiments were aided by the following pharmacological tools: the MR agonist aldosterone (ALDO), the MR antagonists spironolactone (SPIRO) and RU28318 (oxprenoate), and the GR agonist DEX. Exposure of cultures to DEX at a dose of $10^{-5}$ M led to a significant loss of mature (MAP-2 positive) neurons, an event accompanied by a significant increase in the incidence of apoptosis. The results from these studies involving a system in which DEX can directly access hippocampal cells, therefore demonstrate that DEX has the intrinsic potential to induce neuronal cell death, and that its effects occur independently of the HPA axis and other confounding factors. Consistent with previous results which suggested that low, MR-activating doses of corticosterone in rats (*in* vivo) may be neuroprotective (Hassan *et al.*, 1996; Sousa *et al.*, 1999; Almeida *et al.*, 2000), it was observed here that the apoptotic actions of DEX could be significantly attenuated when the MR agonist ALDO ($10^{-5}$ M) was added to the culture medium. Since ALDO on its own did not alter apoptotic cell and neuronal numbers, our results add currency to the view that MR can trigger neuroprotective mechanisms; this interpretation is supported by reports from other laboratories that either ALDO or low levels of corticosterone prevent or reverse ADX-induced apoptosis (Woolley *et al.*, 1991; Sloviter *et al.*, 1993; Hu *et al.*, 1997). In light of the results showing the ability of ALDO to counteract the apoptotic actions of DEX, and the fact that our culture medium contained low levels of cortisol (which has pharmacological properties and biological actions

which are comparable to those of corticosterone), it was hypothesized that the blockade of 'medium-activated' MR would accentuate the effects of GR stimulation. Analysis of apoptosis after concomitantly treating cultures with either SPIRO or RU28318 and a sub-optimal dose of DEX ($10^{-6}$ M) proved our hypothesis correct – whereas DEX at a dose of $10^{-6}$ M did not induce cell death, its combination with a range of doses of SPIRO or RU28318 ($10^{-8}$-$10^{-5}$ M) significantly exacerbated the occurrence of neuronal apoptosis. Besides acting on MR, SPIRO can also antagonize androgen and progesterone receptors (Nirdé *et al.*, 2001); since similar results were however obtained with the more receptor-selective drug RU28318, the inference that neuroprotective effects are mediated by MR appears to be warranted.

Together, results obtained using a cellular model provide clear evidence that glucocorticoids can lead to hippocampal cell death without the participation of other aggravating factors (e.g. excitotoxins), so long as their effects are not masked by previously-activated MR. The presented results also bolster the view previously advanced by this laboratory that hippocampal neuronal survival depends on the tonic occupation of MR, and exemplify that the 'receptor balance hypothesis,' originally generated on the basis of endocrinological and behavioral data (de Kloet *et al.*, 1998), can be extended to include hippocampal survival and death.

C. NMDA regulation of neuronal survival *vs.* death depends on site of action of NMDA

As mentioned in the General Introduction (Chapter 1), glucocorticoids and GluR share an intimate relationship. Previous work showed that GluR stimulation can result in excitotoxicity and that the latter can be amplified by pre-treatment with glucocorticoids (Goodman *et al.*, 1996; Behl *et al.*, 1997; Abraham *et al.*, 2001; Johnson *et al.*, 2002) prompted us to examine the effects of NMDA pre-treatment on DEX-induced apoptosis (Chapter 4). To do this, we treated hippocampal cultures with NMDA at doses ranging from 1 to 10 μM for a brief period (15 min) before exposure to DEX (1 μM) for 72 h. We observed a complete abolition of the apoptotic actions of DEX when cells were pre-exposed to NDMA at 1 and 5 μM; at a dose of 10 μM, NMDA resulted in a rapid necrotic response.

The NMDA.R represents the most-intensely studied GluR, especially in the context of neurotoxicity. These receptors are heteromeric in nature, consisting of a common NR1 subunit and one or more NR2 subunits (NR2A-D), whose insertion in the NMDA.R complex varies during development and maturity of synapses (Li *et al.*, 1998; Stocca and Vicini, 1998; Tovar and Westbrook, 1999). For example, it is known that whereas NR2A is predominantly found in NMDA.R located at the *synapse*, NR2B features almost exclusively in NMDA.R at *extrasynaptic* (non-synaptic) sites. An important concept that has emerged from recent studies is that *synaptic* NMDA.R can initiate neuroprotective mechanisms; in contrast, activation of *extrasynaptic* NMDA.R results in cell death (Hardingham *et al.*, 2002). The results reported in Chapter 4 demonstrate that selective inhibition of NR2B with ifenprodil results in a suppression of DEX-induced apoptosis; this finding is consistent with the documented efficacy of ifenprodil in retarding cell death in various animal models of neurodegenerative disease (for review, see Chenard and Menniti, 1999). Thus, blockade of *extrasynaptic NMDA.R* can counteract the apoptotic actions of glucocorticoids.

In addition to the above experiments, studies of the other important ionotropic glutamate receptor AMPA.R were also undertaken. Selective blockade of AMPA.R with NBQX also proved effective at attenuating DEX-induced apoptosis in hippocampal cultures. Further, the present studies revealed a role for metabotropic glutamate receptors (mGluR) insofar that E4CPG and CPPG, selective antagonists of mGluR I/II and II/III,

respectively, were able to rescue hippocampal cells from DEX-induced apoptosis at very low doses. In general, the manifestations of mGluR-mediated actions are slow and involve gene activation. Type I mGluR have been associated with neuronal death while Type II/III mGluR have been shown to contribute to neuronal survival (Snyder *et al.*, 2001; Allen *et al.*, 2001; Miskevich *et al.*, 2002; Heidinger *et al.*, 2002). Together, these findings suggest the involvement of either complex regulatory interactions between the various mGluR or the upregulation of neurotoxic type I mGluR by DEX.

The results reported in this section represent the first attempt to understand the mechanistic and functional nature of interactions between GluR and glucocorticoids. They demonstrate that the apoptotic actions of DEX are at least partly mediated by GluR of the NMDA and metabotropic types. In addition, the results reported herein show that low doses of NMDA, acting via *synaptic* NMDA.R can effectively block hippocampal cell death induced by DEX. Lastly, this work indicates that glucocorticoids can cause apoptosis in hippocampal cells by triggering rapid (NMDA.R-mediated) as well as slow (mGluR- or GR-mediated) responses, and that the final outcome of glucocorticoid treatment on hippocampal cell survival depends on the convergence and integration of transcriptional signals (e.g. GluR and agonist availability; regulation of apoptosis-related genes) and signals originating at the cell membrane (e.g. $Ca^{2+}$ conductance).

### 5.3. Mechanisms underpinning glucocorticoid, NMDA and cytokine actions on neural development and survival

A. Glucocorticoid-glutamate interactions

Glucocorticoids can exert rapid, transient effects on neuronal excitability involving increased cytosolic concentrations of $Ca^{2+}$ (Nair *et al.*, 1998). However, glucocorticoid actions are best known to be mediated through GR which are transcription factors; our laboratory has previously elucidated some of the cell death-related molecular pathways triggered by exposure to the potent GR agonist DEX (Almeida *et al.*, 2000). Thus, glucocorticoid effects on hippocampal structure and function do not necessarily result from one exclusive mechanism (electrophysiological *vs.* genomic) but may rather occur as a result of integrated signals arising from both the cell membrane and transcriptional activity. For example, by influencing the transcription of $Ca^{2+}$ channel and $Ca^{2+}$ extrusion pump genes (Bhargava *et al.*, 2000), glucocorticoids contribute to long-term alterations in the dynamics of intracellular $Ca^{2+}$ levels, including those originating at the plasma membrane (Kerr *et al.*, 1992; Elliott and Sapolsky, 1993; Nair *et al.*, 1998; Bhargava *et al.*, 2000).

Exaggerated and chronic elevations in intracellular $Ca^{2+}$ accompany glutamatergic excitotoxicity (Choi, 1991; Coyle and Puttfarcken, 1993) and have also been proposed to at least partially underlie the neurotoxic effects of glucocorticoids (Joëls, 2001). Increases in cytosolic $Ca^{2+}$ concentrations result from the activation of both ionotropic glutamate receptors (iGluR) and mGluR, albeit through different mechanisms: iGluR stimulate the influx of $Ca^{2+}$ from the extracellular space, whereas mGluR mobilize $Ca^{2+}$ from intracellular reservoirs (Maiese *et al.*, 1999; Otani *et al.*, 2002). As mentioned before, glucocorticoids can increase NMDA.R expression and glutamate synthesis and accumulation (Weiland *et al.*, 1997; Moghaddam *et al.*, 1994), and potentiate glutamate-induced cell death (Goodman *et al.*, 1996; Behl *et al.*, 1997; Abraham *et al.*, 2001; Johnson *et al.*, 2002). GluR are well-recognized triggers of neuronal cell death and may occur either acutely upon activation or after a period of delay (Choi, 1991). Apoptotic and necrotic cell death are distinguishable on the basis of their morphological characteristics and ionic dependence. The rapid-onset form is necrotic in nature (cell swelling and ultimate cell lysis). In contrast, the delayed form is $Ca^{2+}$-dependent, and is accompanied by cell shrinkage,

nuclear condensation and fragmentation; all of the latter features are typical of apoptosis (Choi, 1991). In the work described in Chapter 4, the dominant form of cell death observed following treatment of hippocampal cells with either NMDA or DEX was of the apoptotic type.

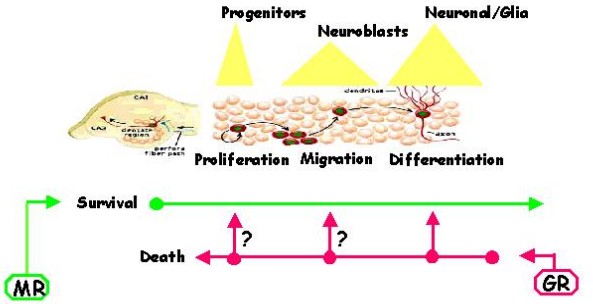
On the other hand, low doses of NMDA have been shown to elicit neurotrophic and anti-apoptotic mechanisms in neurons (Marini *et al.*, 1998; Resink *et al.*, 1996; Brandoli *et al.*, 1998); moreover, one of the implicated neurotrophins, brain-derived nerve growth factor (BDNF), was found to stimulate (pro-survival) NR2A subunit expression and to suppress NR2B (death-promoting) expression (Glazner and Mattson, 2000). Because the experimental paradigm used in Chapter 4 involved chronic exposure to DEX, it is pertinent to mention that GR activation was previously found to upregulate NR2B subunit gene expression with a concomitant decrease in the expression of the gene encoding NR2A subunit (Nair *et al.*, 1998).

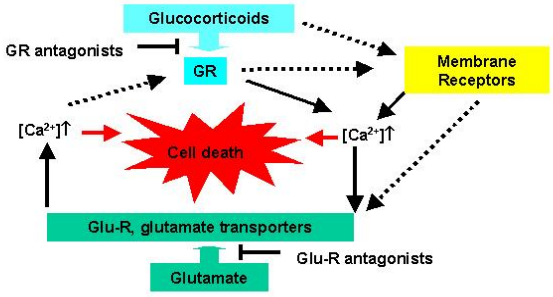
As noted above, NMDA.R which include the death-promoting NR2B subunit are predominantly localized at *extrasynaptic* sites; in contrast those comprising the pro-survival NR2A subunit have a synaptic location (Riccio and Ginty, 2002). Since the appearance of NR2A- and NR2B-containing NMDAR is correlated with the ontogeny of synapses (Tovar and Westbrook, 1999; Li *et al.*, 1998; Stocca and Vicini, 1998), it is pertinent to note that although the cultures used for the present studies were relatively young (experiments carried out after 6 days *in vitro*), cell-cell interactions were abundantly evident at the light microscopic level. In order to distinguish between *synaptic* and *extrasynaptic* NMDA.R in the mediation of the protective actions of low doses of NMDA against DEX-stimulated apoptosis, the recently described elegant pharmacological paradigm described by Hardingham *et al.* (2002) was adopted. Briefly, the paradigm which consists of briefly (15 min.) pre-treating hippocampal cultures with the GABA antagonist bicuculline (to activate synaptic GluR) and MK801 (to block active NMDAR) prevents NMDA activation of neuroprotective signaling cascades, leaving only extrasynaptic NR2B-containing NMDAR available for NMDA binding. In the studies described in Chapter 4, bicuculline/MK801-pretreated cultures were exposed to 1 or 5 μM NMDA and DEX before analysis of apoptosis using the TUNEL assay. The observation that the bicuculline/MK801 pre-treatment abrogated the ability of NMDA to oppose the apoptotic actions of DEX is consistent with a synaptic site of NMDA-induced neuroprotection (Hardingham *et al.*, 2002).

In an experiment to examine the impact of NR2B subunit-containing NMDAR blockade on the neuroprotection afforded by low doses of NMDA against DEX-elicited apoptosis, NR2B blockade was achieved by pre-treating cells with NMDA and ifenprodil (Chapter 4). The rationale was that this design would ensure binding of ifenprodil (an activity-dependent antagonist), to *extrasynaptic* receptors and, at the same time, permit (or even enhance) the activity of synaptic NMDAR. Contrary to expectations, we observed NR2B blockade amplified the degree of apoptosis that was seen after DEX treatment alone. In the absence of supporting evidence to the contrary, we tentatively propose that since the NMDA/ifenprodil pre-treatment was transient (i.e. drugs were washed out after 15 min.), the effects observed reflect changes in the sensitivity and/or synthesis of NR2B-containing (death-promoting) receptors; this could include upregulation of NR2B during the subsequent exposure (72 h) to DEX alone. Other likely events include pre-treatment- or DEX-induced downregulation of NR2A-containing (pro-survival) NMDA.R. Another plausible explanation would be that by employing transcriptional mechanisms to elevate glutamate synthesis (cf. Ábrahám *et al.*, 1996), DEX would effectively make more neurotoxic glutamate available to NMDA.R (containing NR2B subunits), thus positively driving a vicious circle. These findings showing the involvement of NMDA.R in the apoptotic actions of DEX, do not

necessarily exclude the possibility of direct, GR-mediated, apoptotic actions of DEX, as reported in Chapter 3. A model summarizing our views on how glucocorticoids and glutamate receptors interact to regulate the balance between hippocampal cell survival and death is depicted in the following figure.

Based on figure by Jacobs B.L.
http://www.americanscientist.org/content/AMSCI/AMSCI/Image/FullImage_200342795432_307.jpg



B. Pathways mediating TGF-β2 and BDNF signaling

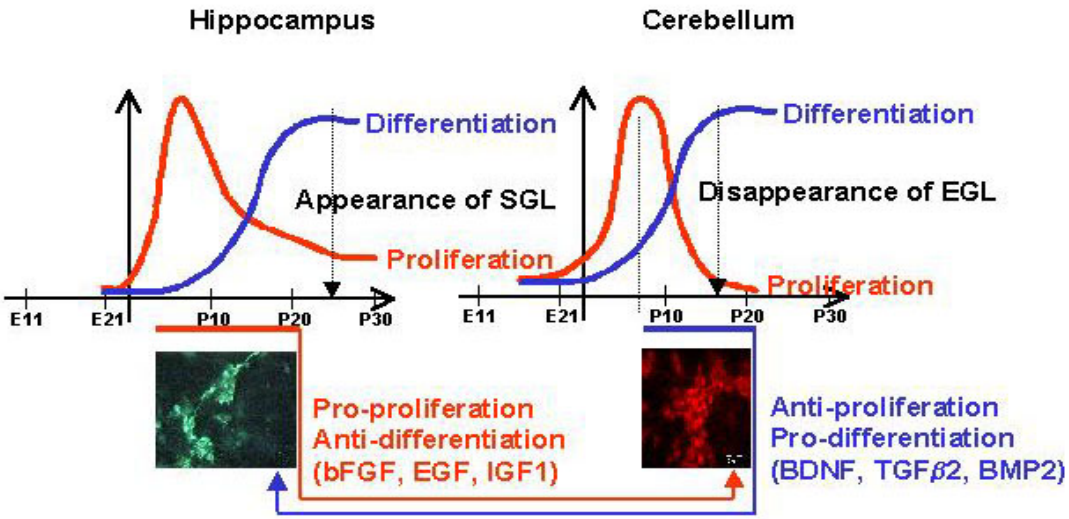
Studies focused on verifying the role of BDNF in the observed $CM_{Cerebellum}$-induced effects on hippocampal cell development (Chapter 2) included immunocytochemical demonstration that BDNF is strongly expressed in cerebellar cultures, but only weakly in hippocampal cultures. Treatment of hippocampal cultures with exogenous BDNF resulted in an inhibition of BrdU uptake and an increase in neuritic length as well as an increased MAP2ab-positive neuronal population. Transfection of cells with a BDNF-expressing plasmid provided similar results on BrdU incorporation. BDNF effects on neuronal differentiation are known to be mediated through TrkB receptors (Klein *et al.*, 1991) and, depending on the strength and duration of the stimulus, BDNF either promotes or inhibits neuronal proliferation by activating the Trk-MEK-ERK pathway (Marshall, 1995; Du *et al.*, 2003). The latter prompted us to examine whether TrkB or ERK signaling is involved in the biological actions of $CM_{Cerebellum}$, i.e. those ascribed to BDNF. Consistent with the literature, we observed that whether TrkB or ERK signaling is involved in the biological actions of $CM_{Cerebellum}$, i.e. those ascribed to BDNF. Consistent with the literature, we observed that blockade of TrkB by transfecting a dominant negative TrkB construct resulted in an attenuation of the pro-neuronal actions of $CM_{Cerebellum}$. In addition, blockade of MEK1/MAPK with UO126 also negated the actions of $CM_{Cerebellum}$, indicating the operation of more than a single mechanism or the convergence of several signaling pathways upon activation by a single ligand. These data contrast with previous findings that activation of ERK1/2 blocks the Smad pathway in *Xenopus* embryos (Pera, 2003); at present, we lack a plausible explanation for this discrepancy. Interestingly, however, as described in Chapter 2, BDNF treatment was shown to elicit a response from a TGF-β reporter gene (3TP-Lux), and to mobilize Smad2 and Smad4, two key players in TGF-β signaling, from the cytoplasm to the nucleus in neuronal cultures. Thus, we tentatively hypothesize that BDNF may be able to activate the Smad pathway through ERK1/2-dependent phosphorylation through as yet undefined mechanisms. It is pertinent to note that other recent studies also alluded to BDNF-TGF cross-talk (Lutz *et al.*, 2004) or interdependence/synergism (Unsicker and Strelau, 2000).

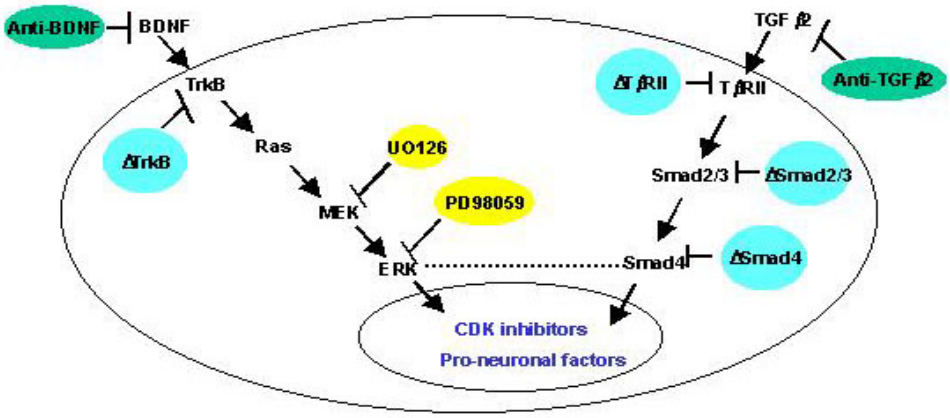
Members of the TGF-β superfamily signal by sequentially binding to two TGFβ receptors (TGFβR) which are transmembrane protein serine/threonine kinases. In the case of TGF-β2, ligand binding to TGFβR-II activates TGFβR-I; both the developing and rat adult hippocampus express TGFβR-II mRNA (Böttner *et al.*, 1996). Receptor-regulated SMAD proteins (R-SMADs) serve as TGFβR-I substrates which, upon phosphorylation, subsequently bind Co-Smad4 and translocate to the nucleus where they form a transcriptionally-active complex after association with DNA-binding partner(s). This last complex binds to promoter elements of target genes whose functions include regulation of the cell cycle, differentiation and cell adhesion, positioning, and movement (Moustakas *et al.*, 2001; Chang *et al.*, 2002; Shi and Massagué, 2003).

TGF-β is a well-known inhibitor of proliferation, inducing cell cycle arrest by suppressing the oncogene Myc, a repressor of the cyclin-dependent kinase inhibitors, p21 and p27 (Seoane *et al.*, 2002; Gartel and Shchors, 2003). In support of the view that the anti-mitogenic effects of $CM_{Cerebellum}$ may be accounted for by TGF-β2 in the CM, we observed an upregulation of p21 and p27 after $CM_{Cerebellum}$ treatment of proliferating hippocampal neurons (Chapter 2). Additional evidence for a key role of TGF-β2 in the hippocampal cell fate-determining actions of $CM_{Cerebellum}$ was obtained in a series of experiments focused on the TGF-β signal-propagating SMAD proteins. Of the various SMAD proteins, Smad2 and Smad 3 mediate TGF-β signals. Smad4 is a requisite partner for transcriptional activity of Smads2 and 3 (as well as Smads1, 5 and 8 which are BMP substrates); the generation of specific downstream responses is thought to depend on the formation of specific R-SMAD-

Smad4 complexes which then recruit different sequence-specific DNA-binding factors (Massagué and Wotton, 2000). Data reported in Chapter 2 demonstrate that $CM_{Cerebellum}$ induces nuclear translocation of EGFP-fused Smad2 and Smad4. Results showing essential roles for Smad3 and Smad4, based on expression of the dominant negative forms, are also presented: these manipulations prevented the transactivation of 3TP-Lux by $CM_{Cerebellum}$ and abrogated the anti-proliferative and pro-differentiating effects of $CM_{Cerebellum}$ in hippocampal cultures. Further support for the notion that TGF-β2, at least partially, accounts for the anti-proliferative activity present in $CM_{Cerebellum}$ is provided by the observation that expression of a vector containing a dominant negative form of TβRII in either primary hippocampal cells or a hippocampus-derived cell line (Hib5) results in a abolition of the $CM_{Cerebellum}$-induced effects on BrdU incorporation and 3TP-Lux reporter activity compared to control.

A model showing the mechanisms underlying BDNF and TGF-β2 control of proliferation and differentiation of hippocampal and cerebellar granule cells is presented in the scheme shown below.



### 5.4. Summary and future directions

The aims of the work undertaken for this thesis may be summarized as follows:

- To identify factors which may be responsible for the differential developmental patterns followed by granule neurons in the hippocampus and cerebellum, and to analyse their signaling pathways (Chapter 2);
- To examine the mechanisms underlying glucocorticoid-induced apoptosis in the hippocampus, in particular with regard to the role of glutamatergic transmission (Chapters 3 and 4).

With respect to the first aim, the principal findings (Chapter 2) show that:

- TGF-β2, supported by the Smad signaling machinery, plays a key role in determining hippocampal cell fate by inhibiting proliferation and, in parallel, inducing neuronal maturation.
- BDNF, better known for Trk receptor-mediated promotion of neurogenesis and differentiation, exerts anti-proliferative and pro-neuronal effects on developing hippocampal neurons by activating a MAP kinase cascade which ultimately converges onto the TGF-β signaling pathway.

Results presented in Chapter 3 show that:

- The glucocorticoid dexamethasone (DEX) can directly induce apoptosis in hippocampal cell cultures *via* the mediation of GR so long as neuroprotective MR are not occupied. However, the actions of glucocorticoids can be modulated depending on the prevailing state of glutamatergic transmission (Chapter 4, see below).
- Suggestions in the literature that the apoptotic effects of DEX reflect the effects of 'chemical adrenalectomy' are therefore not tenable.
- Mature, rather than immature, granule neurons are targeted by glucocorticoids for apoptosis.

Studies on the likely interaction between glucocorticoids and glutamatergic transmission (Chapter 4) revealed that:

- The apoptotic actions of DEX are, at least partly, mediated by GluR of the NMDA and metabotropic types.
- Low doses of NMDA, acting *via synaptic* NMDAR can effectively block hippocampal cell death induced by DEX.
- Gluccorticoids can cause apoptosis in hippocampal cells by triggering rapid (NMDA.R-mediated) as well as slow (mGluR- or GR-mediated) responses. The final outcome of glucocorticoid treatment on hippocampal cell survival depends on the convergence and integration of transcriptional signals and signals originating at the cell membrane.

Here follow some considerations which may be pertinent to attempts to integrate the novel observations made in this work and to identify further lines of investigation.

- Both the TGF-β and glucocorticoid signaling pathways contribute to the regulation of a variety of neurodevelopmental and physiological processes. For example, this work demonstrates that both factors inhibit proliferation of hippocampal granule cell progenitors, and other studies have demonstrated that they are both inducers of apoptosis in a variety of cell types (Schmidt *et al.* 2004; Zalavras *et al.*, 2003; Druilhe *et al.*, 2003; Lee *et al.*, 2002; Siegel and Massague, 2003; Schuster and Krieglstein, 2002). These observations suggest that a certain degree of interaction between these pathways must exist in order to ensure manifestation of coordinated responses. Indeed, GR have been reported to inhibit TGF-β signaling

by directly targeting Smad3 in Hep3B cells and COS cells (Song *et al.*, 1999; Li *et al.*, 2003). On the other hand, TGF-β can block GR signaling through AP-1-mediated transcriptional repression in mouse fibrosarcoma L929 cells (Periyasamy and Sanchez, 2002). At the same time, glucocorticoids upregulate TGF-β mRNA expression in various cell types (AyanlarBatuman *et al.*, 1991; Boulanger *et al.*, 1995; Reyes-Moreno *et al.*, 1995; Wang *et al.*, 1995; Oursler *et al.*, 1993) while the anti-proliferative effects of glucocorticoids are abrogated by TGF-β neutralizing antibodies (AyanlarBatuman *et al.*, 1991; Johnson *et al.*, 1993; Reyes-Moreno *et al.*, 1995). To what extent these findings apply to the regulation of neurodevelopmental processes remains to be elucidated.

- With respect to their mechanisms of action, recent evidence suggests that glucocorticoids can also act through pathways independent of binding of GR to glucocorticoid response elements (GRE) function through GRE (Di *et al.*, 2003; Qiu *et al.*, 2003; Evans wt al., 2003); similarly, apparently not all TGF-β actions are mediated through Smad pathways (Massague and Chen, 2000). It will be interesting to identify whether TGF-β and glucocorticoids share common signaling pathways which result in the inhibition of neurogenesis and stimulation of neuronal differentiation and/or apoptosis; in addition, their utilization of GRE- and Smad-dependent and/or GRE- and Smad-independent mechanisms will be worth investigating.
- The first evidence of cross-talk between TGFβ and BDNF in the control of hippocampal granule cell maturation deserves more thorough analysis. In particular, it will be important to know whether the ability of BDNF to enhance the Smad signaling pathway results from its stimulation of ERK1/2 or indirectly, increased TGFβ secretion. Further, clarification is needed as to how ERK1/2 stimulates (rather than inhibits) Smad activity, and interactions of TGFβ with MAPK cascades also need further investigation.
- Although TGFβ and BDNF were here identified as key molecules in the control of granule cell development, the involvement of other factors which might also fulfil such roles cannot be excluded. The application of more efficient CM screening (e.g. 2-D gel electrophoresis and MS-MS approaches and/or antibody array assays, will greatly facilitate the search for other candidates.
- Recent studies have identified GR in mitochondria, organelles which are intimately involved in the apoptotic process (Koufali *et al.*, 2003; Demonacos *et al.*, 1995, 1996). The role of mitochondrial GR in DEX-induced neuronal cell death, conventionally ascribed to nuclear GR, deserves evaluation.

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**Annex I**

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# Gradients and hemispheric asymmetries in cell proliferation and apoptosis in the dentate gyrus

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**Summary**

Cell proliferation and death occur contemporaneously in the hippocampal dentate gyrus. On the premise that the positioning and survival of newly-born neurons are important determinants of hippocampal function, we here analyzed the topographical distribution of cell proliferation (bromodeoxyuridine incorporation) and apoptosis (morphological criteria and TUNEL histochemistry) in 1-month old rats. Our analysis included comparisons between left-right hemispheres, dorsal-ventral hippocampus and suprapyramidal-infrapyramidal blades of the dentate gyrus; since newly-generated cells appear in the subgranular zone (SGZ) before migrating inwards, we also compared the granular cell layer (GCL) *versus* SGZ. Highest proliferation indices were found in the SGZ (47%), followed by the GCL (29%), hilus (16%) and molecular layer (8%). Whereas proliferation rates did not differ between hemispheres, analysis along the rostro-caudal axis revealed higher cellular division in the ventral SGZ. In addition, gradients of proliferation were observed in the subdivisions of the GCL with the external, infrapyramidal and ventral zones showing highest proliferative potential. The total number of apoptotic cells was evenly distributed throughout the dentate gyrus, although the incidence of apoptosis decreased gradient-wise from the tip of the suprapyramidal layer and was highest in the external third of the GCL and lowest in its internal third, in marked contrast to the proliferation profile. Curiously, more apoptosis was observed in the left hippocampus. This study shows that neurogenesis and apoptosis occur in specific 'niches' and provides a useful basis for future investigations into the relationship between hippocampal structure, function and plasticity.

## Introduction

New neurons are continually generated from a local population of stem or progenitor cells in the dentate gyrus of the hippocampus (Altman and Das, 1965; Cameron *et al.*, 1993; Kuhn *et al.*, 1996). Our previous stereological studies indicated that the total number of hippocampal granule cells increases by some 25% (ca. 250,000 cells) during the first 6 months of life and by some 5% during the subsequent 6 months (Sousa *et al.*, 1999, 2000); these observations are commensurate with data showing that the rate of granule cell acquisition declines with increasing age (e.g. Kuhn *et al.*, 1996; Kempermann *et al.*, 1998; Cameron and McKay, 1999, 2001; Nacher *et al.*, 2003).

The cytoarchitecture of the dentate gyrus ultimately results from neurogenesis and the concurrent physiological elimination of neurons through apoptosis (Gould *et al.*, 1991; Lossi and Merighi, 2003). Interestingly, neurogenesis and neuronal apoptosis share a number of common regulatory factors, e.g. adrenal steroids and excitatory amino acids (Gould and McEwen, 1993; Cameron and Gould, 1994). This, together with the aforementioned temporal overlap strongly suggests that the two processes are intimately related and that the balance between them determines the rate of postnatal granule cell turnover. Despite numerous studies on neuronal birth and death in the postnatal dentate gyrus, there is a conspicuous lack of information with respect to the topographic distribution of proliferating and dying neurons (e.g. rostro-caudal, inter- and intra-layer and hemispheric asymmetry). Such maps could help promote our understanding (i) of why individual granule cells show differing electrophysiological properties (Wang *et al.*, 2000), (ii) how specific functions result from the suprapyramidal vs. infrapyramidal and intra-layer positions of individual granule cells (Scharfman *et al.*, 2002), (iii) what structural and neurochemical mechanisms operating during dentate development provide the ventral and dorsal hippocampus with functionally-distinct properties (Moser and Moser, 1998) and (iv) whether hemispheric asymmetry in granule cell turnover accounts for hemispherical differences in hippocampal volume and function in both rodents (Verstynen *et al.*, 2001) and humans (Zaidel *et al.*, 1998; Utsunomiya *et al.*, 1999).

In order to determine whether neuronal proliferation and death in the hippocampus occur according to subregion-specific patterns or in a stochastic fashion, we undertook a systematic stereology-based mapping of these events in young (1 month old) rats under basal conditions. Cell proliferation was assessed immunohistochemically after incorporation of the thymidine analogue bromodeoxyuridine (BrdU) into the DNA of cells in S-phase; apoptosis was evaluated using established morphological criteria coupled with terminal deoxynucleotidyl transferase-mediated dUTP-nick-end labeling (TUNEL) histochemistry. Disturbed granule cell proliferation is increasingly being proposed as one of the neurobiological mechanisms responsible for psychiatric and neurological disorders (Jacobs *et al.* 2000; Duman *et al.*, 2001; Eisch, 2002). However, only one previous study (Ekdahl *et al.*, 2001) has addressed the issue of likely concomitant cell death and none has studied subregion-specific changes in neuronal turnover. The information gained here will allow more critical analysis and exploitation of the factors directing birth and functional organization of dentate neurons.

## Materials and Methods

### Animals

Six male Wistar rats (Charles River, Barcelona, Spain), aged 4 weeks and with a mean weight of 120g, were used in this study. Animals were housed under standard laboratory conditions (12 hours light cycle; 22ºC, 55% humidity; food and water available *ad libitum*). Experiments were conducted in accordance with local regulations (European Union Directive 86/609/EEC) and NIH guidelines on animal care and experimentation.

### Tissue preparation

Rats received daily intraperitoneal injections of 50 mg/kg BrdU (Sigma, St. Louis, MO) on 3 consecutive days and were killed by rapid decapitation 24 hours after the last injection. Brains were carefully removed, placed in cryoprotectant and snap-frozen in liquid nitrogen. Serial coronal sections (20 μm), extending over the entire length of the hippocampus, were obtained using a cryostat and mounted on poly-L-lysine-coated slides.

### Histochemical procedures

*BrdU labelling*

BrdU incorporation was detected by immunocytochemistry on every 8$^{th}$ section (Fig. 1A). Briefly, sections were fixed in 4% paraformaldehyde (PFA) for 30 minutes, permeabilized for 10 minutes in a solution containing 0.2% Triton X-100 in Tris buffer saline (TBS), microwaved (20 minutes) while immersed in citrate buffer (0.1 M), and acidified in 2 M HCl (30 minutes). Endogenous peroxidase activity was blocked with 3% $H_2O_2$ in TBS (10 minutes) and non-specific staining was blocked with 4% bovine serum albumin (BSA) in TBS (30 minutes). Subsequently, sections were incubated overnight with a mouse monoclonal anti-BrdU (1:100, Chemicon, Temecula/California). Antigen visualization was carried out using a universal detection system (BioGenex, San Ramon, CA) and diaminobenzidine (DAB: 0.025% and 0.5% $H_2O_2$ in Tris-HCl 0.05M, pH 7.2). Specimens were lightly counterstained with hematoxylin.

*TUNEL*

One out of eight sections containing the hippocampal formation was used for TUNEL histochemistry (Fig. 1B). Sections were fixed as described above, permeabilized in a two-step procedure (0.1% trypsin in PBS, pH 7.2 at 37 ºC, followed by 0.1% Triton X-100 in PBS, 5 minutes at room temperature) and treated with 3% $H_2O_2$ in PBS (3 minutes) to block endogenous peroxidases. Sections were then pre-incubated in terminal deoxynucleotidyl transferase (TdT) buffer before incubation (37º C, 1 hour) in a cocktail consisting of 13.5 μL TdT (MBI Fermentas, Hanover/MD *)*, 6.75 uL dUTP-Biotin (Roche, Basel/Switzerland*)*, 90 μL TdT buffer, 20 μL TdT enzyme buffer (MBI Fermentas) and 770 μL distillate water. Development and visualization was achieved using a commercial avidin-biotin/DAB system (Vector Labs, Burlinghame, CA). Hematoxylin was used as a counterstain.

### Stereological procedures

Estimates of cell density in the different subdivisions of the hippocampus were obtained using the optical fractionator method in combination with the *StereoInvestigator* software (MicroBrightField, Williston/VT). Briefly, each 8$^{th}$ section was used for sampling the different areas of interest. Starting at a random position, visual fields were sampled by step-size movements of 150 μm in the x-axis and 200 μm in the y-axis for all subdivisions analyzed, except the hilus (Hl) and molecular layer (MCL) in which the step-size was 350 μm in both axis. Counting frames areas were 625 $\mu m^2$ at tissue level. Cells were counted under a x100 lens, thus obtaining a resolution sufficient to easily identify the different layers and cells types. Cellular discrimination

was based on morphological and staining criteria: apoptotic cells were identified by TUNEL coloration and, in addition, by their small size, chromatin condensation, and vesicular formation; cells that were positively labeled with BrdU were considered as cells which had been recently generated, i.e. within the timeframe of the experiment.

The following criteria were used to define transversal divisions within the granular cell layer (GCL) and subgranular zone (SGZ): the angular subdivision (Ang) was considered as the area extending from the tip of the crest up to the end of its curve; the remaining area was divided into two equal parts, delineating the intermediate (Med) and extreme (Ext) subdivisions (Fig. 1C). These subdivisions were further distinguished in terms of their position within the suprapyramidal (Supra) and infrapyramidal (Infra) blades. Longitudinal divisions of the GCL (internal, intermediate and external) were defined by dividing the thickness of the GCL into 3 equal parts. The SGZ was considered to be a 4-cell-body-wide zone at the border between the GCL and Hl. The division between dorsal and ventral hippocampus was based on the relative position of the dentate gyrus with respect to the third ventricle or cerebral aqueduct: areas located superior to a transverse plane passing at the top of these structures were considered dorsal, whereas those located inferior were considered ventral.

### Statistical analysis

Results are expressed as mean $\pm$ SEM. SEM and coefficient of error (CE) were calculated accordingly to Gundersen and Jensen (1987). Data were analyzed using one- or two-way analyses of variance (ANOVA), followed by Tukey *post-hoc* multiple comparisons. Differences were considered statistically significant if $p \leq 0.05$.

## Results

### Volumes of the dentate gyrus

Analysis of the volumes of the different layers of the left dentate gyrus revealed a slight, but not significant ($F_{(7,47)}$= 0.097; p=0.8), predominance of the left dentate gyrus (Table 1). Yet, a significant effect of location along the rostral-caudal axis was revealed by ANOVA ($F_{(7,47)}$= 32.1; p≤ 0.0005). Comparisons between dorsal and ventral divisions showed increased volumes in the dorsal component of the molecular cell layer (MCL), granular cell layer (GCL), subgranular zone (SGZ), except in the hilus (Hl) where the volume of the ventral division outweighed that of the dorsal division (Table 1).

## BrdU staining

As illustrated in Fig. 2A, significant differences ($F_{(3,23)}$= 237.8; p≤ 0.0005) were observed in the total number of BrdU-positive cells within the various divisions of the dentate gyrus: molecular cell layer (MCL), granular cell layer (GCL), subgranular zone (SGZ) and hilus (Hl). The ratio of cellular proliferation was as follows: subgranular zone >> granule cell layer >> molecular cell layer = hilus.

Whereas no significant hemispherical differences in the densities of BrdU-incorporating cells were detected in any of the divisions of the dentate gyrus (Fig. 2B), there were significant differences in proliferation along the rostro-caudal axis of this hippocampal subfield ($F_{(3,23)}$= 56.6; p≤ 0.0005). Post-hoc comparisons revealed that mitotic cells predominate in the ventral SGZ (Fig. 2C).

As shown in Fig. 2D, ANOVA revealed a significant effect ($F_{(3,23)}$=416.5; p≤ 0.0005) of location of proliferating cells into supra- and infra-pyramidal blades, as well as in subdivisions of the dentate gyrus ($F_{(3,23)}$= 49.7; p≤ 0.0005). *Post-hoc* comparisons demonstrated a significant increase in the numerical density of BrdU-labeled cells in the infra-pyramidal blade of both areas (Fig. 2D).

Within the GCL, proliferation was higher in the internal third of the cell layer ($F_{(2,15)}$= 238; p≤ 0.0005) (Fig. 2E)].

Analysis of the densities of BrdU-positive cells in the GCL and SGZ sub-divisions revealed a significant effect of location within supra- vs infra-pyramidal blades ($F_{(5,35)}$= 57.9; p≤ 0.0005) and in the transversal position within these blades ($F_{(5,35)}$= 4.3; p= 0.03). A gradient of proliferation increasing from the extreme portion of the suprapyramidal blade to the angular zone of the infrapyramidal blade was found, with a decrease from the angular zone to the extreme portion of this blade (Fig. 2F).

## Apoptosis

As illustrated in Fig. 3A, and confirmed by ANOVA ($F_{(3,23)}$=14.5; p≤ 0.0005), analysis of densities of apoptotic cells between the main divisions of the dentate gyrus showed the GCL and SGZ to have the highest levels of apoptosis (Fig. 3A). In addition, a significant effect of hemispheric location ($F_{(7,47)}$=12.4; p=0.001) was detected by ANOVA; comparisons for potential differences between hemispheres revealed that apoptotic cells were more abundant in the left dentate gyrus, although the differences were significant only in the SGZ (Fig. 3B).

There were no significant differences ($F_{(7,47)}$= 1.3; p=0.27 ) in the occurrence of apoptosis along the rostro-caudal axis of the dentate gyrus (Fig. 3C). ANOVA revealed an effect of location of apoptosis in the supra- and infra-pyramidal blades ($F_{(3,23)}$= 415; p≤ 0.0005), as well as in the subdivisions of the dentate gyrus ($F_{(3,23)}$= 49.5; p≤ 0.0005), with higher levels of apoptosis in the supra-pyramidal blade of the GCL (Fig. 3D). Scrutiny of the sublayers within the GCL revealed an increasing gradient of apoptosis from the internal third to the external third of the GCL ($F_{(2,17)}$= 381.4; p≤ 0.0005) (Fig. 3E). Further, it was found a significant effect of both supra- vs infra-pyramidal blade location ($F_{(5,35)}$=5.0; p=0.003) and transversal position within these blades

($F_{(3,23)}$=3.4; p=0.04) for apoptotic cells; post-hoc comparisons demonstrate that the highest index of apoptosis occurs in the extreme portion of the supra-pyramidal blade (Fig. 3F).

## Discussion

### Granule cell turnover

The postnatal dentate gyrus displays remarkable plastic properties, reflected, for example, by its ability to generate new granule cells from progenitors cells located in the SGZ throughout life (Altman and Das, 1965; Cameron *et al.*, 1993; Kuhn *et al.*, 1996). Despite a decline in proliferative potential with increasing age, postnatal neurogenesis contributes significantly to the number of granule cells, with estimates of an additional 1500-9000 new granule cells being born each day (Biebl *et al.*, 2000; Cameron and McKay, 2001; Fisher *et al.*, 2002) but the net increase in the volume and total cell number of the dentate gyrus during postnatal life is considerably smaller due to the continuous loss of granule cells through apoptotic mechanisms (Sousa *et al.*, 1998, 1999).

The programmed elimination of cells is an essential component of the neural cell turnover process and is important for the shaping and maintenance of brain structures (Raff *et al.*, 1993; Burek and Oppenheim, 1996; Rubin, 1997). Hippocampal granule cells undergo apoptosis during postnatal development and aging and following exposure to various stimuli (Gould *et al.*, 1991; Sloviter *et al.*, 1993; Gould and Cameron, 1996; Hassan *et al.*, 1996; Almeida *et al.*, 2000). Under basal conditions, the rates of neurogenesis and apoptosis show a strong inverse correlation during development and early life, and tend to equalize thereafter. It is thus conceivable that an inappropriate balance in these rates will predispose the individual to pathology, including accelerated aging of the neural substrate and impaired function. Supporting the view that neurogenesis and apoptosis occur in a coordinated and inter-dependent fashion are the facts that the two phenomena occur in tandem and share several intrinsic and extrinsic triggers (e.g. Gould and McEwen, 1993; Cameron and Gould, 1994; Lossi and Merighi, 2003).

The starting premise for this study was that evidence for differential occurrence of these events could eventually contribute to improving understanding of the relationship between structure and function. Accordingly, we here aimed to determine gradients and laterality in the incidence of neurogenesis and apoptosis in the dentate gyrus of the young male rat, rather than to establish the precise daily ratio between neuronal birth and death in this hippocampal area; while the latter would demand a different experimental design because of the complex dynamics of each of these events, the protocol that was used allows comparison with the majority of published studies pertaining to the issue examined in this paper.

### Topographical specificity of proliferation

A major objective of this study was to analyze the precise location and extent of neurogenesis and apoptosis in the dentate gyrus. Our results show that proliferating cells are predominantly located in the SGZ and the adjacent (inner third) GCL, confirming observations by Kempermann *et al.*, (2003). Further, this topographical analysis revealed that the density and total number of newly-generated cells is greater in the ventral than in the dorsal hippocampus, and greater in the infrapyramidal than in the suprapyramidal blade of the dentate gyrus. The prevailing consensus is that newly-generated cells migrate inwards from the SGZ, reaching their final location within a few days-weeks of birth, where they ultimately differentiate (e.g. Kempermann *et al.*, 2003). Assuming a role for paracrine factors and synaptic inputs in differentiation, it is highly plausible that location plays a crucial role in determining the final functional phenotype of newly-born granule cells. Generation of cellular diversity is perhaps the most intriguing question of brain development, and there are numerous examples of phenotypically-similar neurons displaying differing functions, presumably because of subtle differences in topography and the physicochemical characteristics of the niche in which individual cells find themselves (e.g. Green and Juraska, 1985; Claiborne *et al.*, 1990; Scharfman *et al.*, 2002).

Natural and induced postnatal neurogenesis is increasingly being implicated in improved hippocampal function (e.g. see Kempermann, 2002; Korovitskiy and Gould, 2003). In this respect, the positional aspects discussed above increase in importance given that, as the organism ages, new neurons are acquired and incorporated into neuronal networks of ever-growing complexity. The herein reported data provides the first systematic and quantitative evidence for topographical differences in the incidence of granule cell turnover; appreciation of these subtle patterns in the distribution of newly-generated and apoptotic neurons should facilitate a better definition of structure-function relationships under healthy and pathological states.

### Apoptosis as a counterbalance to neurogenesis

The demonstration that the topographic distribution of apoptosis and neuronal proliferation in the dentate gyrus differ significantly was unexpected given that neurogenesis and apoptosis in this area share several common stimuli. In contrast to the distribution of proliferating granule cells, apoptotic cells were predominantly found in the suprapyramidal blade. While adrenalectomy-induced apoptosis was previously shown to mainly occur in this blade (Sloviter *et al.*, 1993; Sousa *et al.*, 1997), the current findings are the first to demonstrate such a skewed distribution under basal conditions, and suggest the particular susceptibility of granule cells in this subdivision to apoptosis. Since apoptosis significantly contributes to neural tissue reorganization (Raff *et al.*, 1993; Burek and Oppenheim, 1996; Rubin, 1997) and may underlie reduced hippocampal volumes in pathological states (Lupien *et al.*, 1999; Honig and Rosenberg, 2000; Friedlander, 2003; Sheline *et al.*, 2003), the role of subregion-specific neuronal loss to the plasticity and function of the hippocampus deserves critical assessment in the future. Although at this age neurogenesis exceeds apoptosis in terms of absolute numbers, apoptosis, driven by changing external and internal conditions, is likely to be an integral component of normal granule cell turnover, ensuring structural stability and appropriate connectivity. Our findings point to the necessity of considering apoptosis when evaluating the potential pathologic and therapeutic roles of neurogenesis.

### Hemispherical asymmetry in incidence of apoptosis

Our observation that neurogenesis occurs to an equal extent in both the left and right hippocampal formations whereas the degree of apoptosis is greater in the left hippocampus bolsters the notion that apoptosis has a significant impact on hippocampal structure and function. Under basal conditions, standard morphometric and magnetic resonance imaging analyses revealed that the right hippocampus of the male rat is thicker (Diamond *et al.*, 1982) and has a greater volume than its left counterpart. Interestingly, these hemispherical differences can be permanently manipulated by exposing rats to certain stimuli during postnatal development (Verstynen *et al.* 2001). Whether the present finding - that there is a greater incidence of apoptosis in the left hippocampus - can explain these differences waits more rigorous testing. Meanwhile, it is pertinent to mention that the loss of a relatively small number of granule cells is reflected in a major decrease in hippocampal volume owing to the concomitant loss of neuritic extensions (Sousa *et al.* 2000).

Asymmetry in hippocampal volumes in humans appears to be a consistent finding; according to one study, up to 91% of healthy subjects have larger right hippocampal formations (Utsunomiya *et al.*, 1999). The potential importance of this asymmetry may be reflected in clinical data which strongly indicate a higher incidence of certain psychiatric disorders (e.g. post-traumatic stress disorder and major depression) in subjects with smaller absolute and normalized left hippocampal volumes (Bremner *et al.*, 1997; Mervaala *et al.*, 2000). Lending support to this are the findings that (i) mood scores correlate negatively with left hippocampal volumes (Villarreal *et al.*, 2002), (ii) the normal left-right differences in hippocampal volume are exaggerated in patients

with affective disorders (Mervaala, *et al*, 2000), and (iii) early life events, which have been causally implicated in psychopathology (Gutman and Nemeroff, 2003; McEwen, 2003), selectively reduce the size of the left hippocampus (Teicher *et al.*, 2003).

## Concluding remarks

The herein reported data serves to alert researchers to the potential importance and functional implications of topographical differences in the incidence of granule neuron proliferation and death in the hippocampus. Further, the reported findings provide a much-needed basis for relating the neurodevelopmental positioning of proliferating and dying cells to the final functional phenotype displayed by individual granule cells.

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# Table

**Table 1.** Volumes of the subdivisions of the dentate gyrus in left and right hemispheres ($mm^3$)

| | Left | | | | Right | | |
|---|---|---|---|---|---|---|---|
| | **Dorsal** | **Ventral** | **Total** | | **Dorsal** | **Ventral** | **Total** |
| **ML** | 2.15 (0.02) | 1.59 (0.06) | 3.74 | **ML** | 2.03 (0.06) | 1.48 (0.07) | 3.51 |
| **GCL** | 0.52 (0.01) | 0.39 (0.02) | 0.91 | **GCL** | 0.51 (0.02) | 0.36 (0.02) | 0.87 |
| **SGZ** | 0.30 (0.01) | 0.22 (0.01) | 0.52 | **SGZ** | 0.28 (0.01) | 0.21 (0.01) | 0.49 |
| **Hl** | 0.64 (0.03) | 0.83 (0.04) | 1.47 | **Hl** | 0.62 (0.01) | 0.80 (0.04) | 1.42 |

Values are expressed as mean (SEM). No significant differences are found between the values of left versus right subdivisions of the dentate gyrus. Comparisons between dorsal and ventral subdivisions of dentate gyrus. Left hemisphere: MCL: $p \leq 0.0005$; GCL: $p=0.001$; SGZ: $p= 0.002$; Hl: $p= 0.008$. Right hemisphere: MCL: $p \leq 0.0005$; GCL: $p \leq 0.0005$; SGZ: $p \leq 0.001$; Hl: $p \leq 0.002$. Mean CE= 0.04.

## Figure legends

## Figures

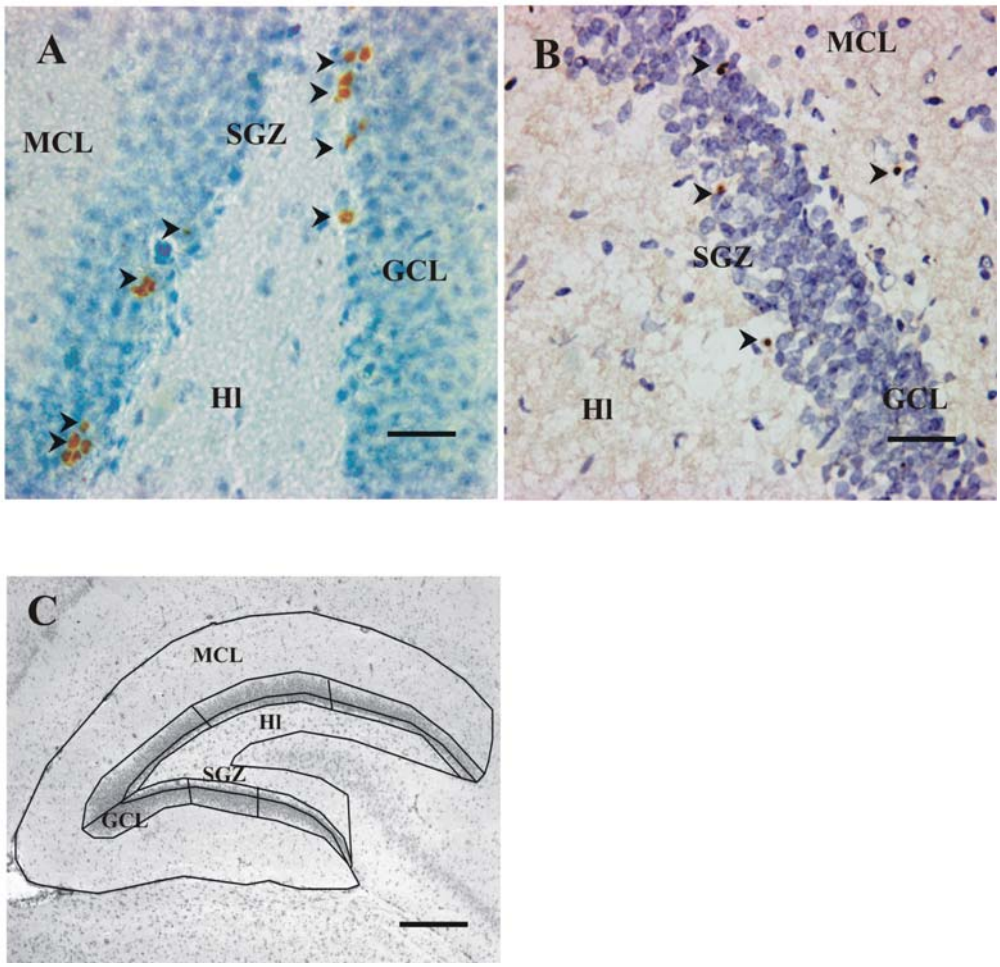


**Fig. 1.** Photomicrographs illustrating the localization of **(A)** BrdU immunoreactive cells (arrowheads) and **(B)** TUNEL positive cells (arrowheads) in dentate gyrus. Scale bar: 50μm. **(C)** Representation of the dentate gyrus divisions including the transversal subdivisions used for the cell counting with the *StereoInvestigator* software. Molecular cell layer (MCL), granular cell layer (GCL), subgranular zone (SGZ) and hilus (Hl). Scale bar: 350μm.

dentate gyrus".

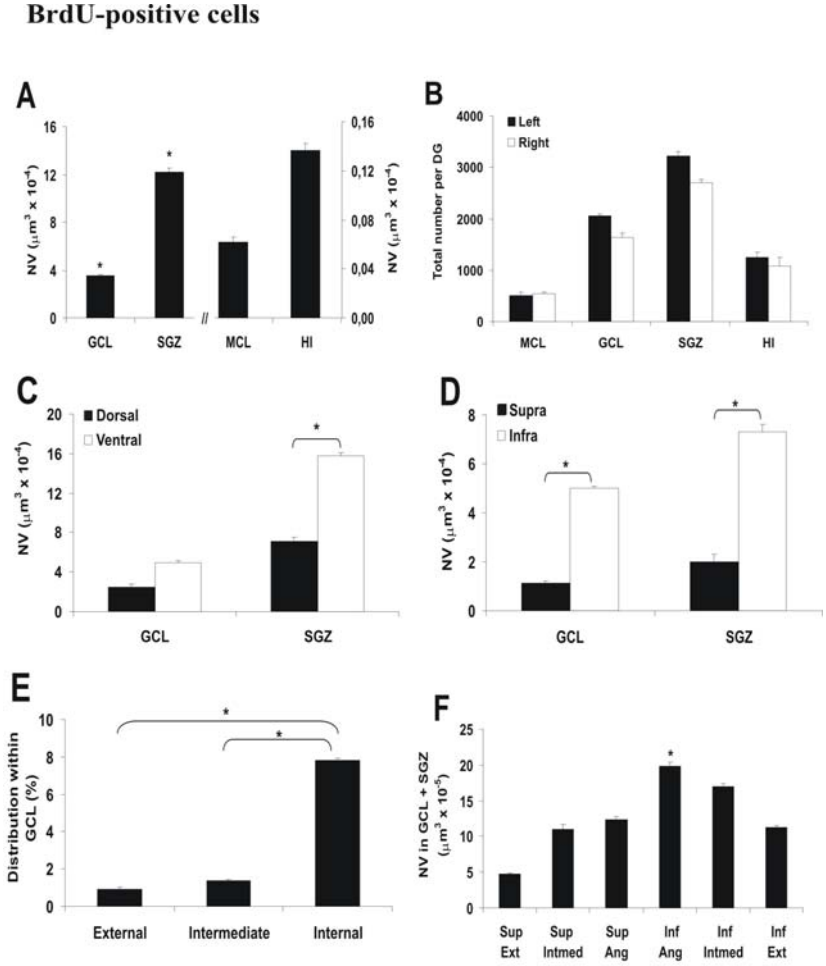


**Figure 2**

**R.Silva et al., 'Gradients and hemispheric asymmetries in cell proliferation and apoptosis in the dentate gyrus'.**

**Fig. 2 (A)** Graphic representation of the distribution of BrdU-positive cells in the dentate gyrus divisions: molecular cell layer (MCL), granular cell layer (GCL), subgranular zone (SGZ) and hilus (Hl). Comparisons among divisions showed SGZ as having the highest levels of BrdU-positive cells followed by GCL. GCL vs MCL: $p \leq 0.0005$; GCL vs SGZ: $p \leq 0.0005$; GCL vs Hl: $p \leq 0.0005$; SGZ vs MCL: $p \leq 0.0005$; SGZ vs Hl: $p \leq 0.0005$. Mean CE=0.04. **(B)** Total number of BrdU-positive cells in left and right dentate gyrus (DG). Mean CE=0.05. **(C)** Comparison between cellular proliferation in dorsal and ventral hippocampus. SGZ: $p \leq 0.0005$. Mean CE=0.05. **(D)** Analysis of the densities of BrdU-positive cells between the supra- (Supra) and infra-pyramidal (Infra) blades. GCL: $p \leq 0.0005$; SGZ: $p \leq 0.0005$. Mean CE=0.02. **(E)** Distribution of BrdU-incorporating cells within GCL longitudinal subdivisions. The highest mitotic levels were found in the internal sublayer. External vs Internal: $p \leq 0.0005$; Intermediate vs Internal: $p \leq 0.0005$. **(F)** Comparison of the densities of BrdU-positive cells between transversal subdivisions of the GCL and SGZ revealed Inf Ang as having the highest levels of mitosis. This sub-division is statistically different from all the others sub-division with the exception of Inf Intmed. Inf Ang vs Sup Ext: $p \leq 0.0005$; Inf Ang vs Sup Intmed: $p \leq 0.0005$; Inf Ang vs Sup Ang: $p \leq 0.0005$; Inf Ang vs Inf Ext: $p \leq 0.0005$. Mean CE=0.03. * $p \leq 0.005$

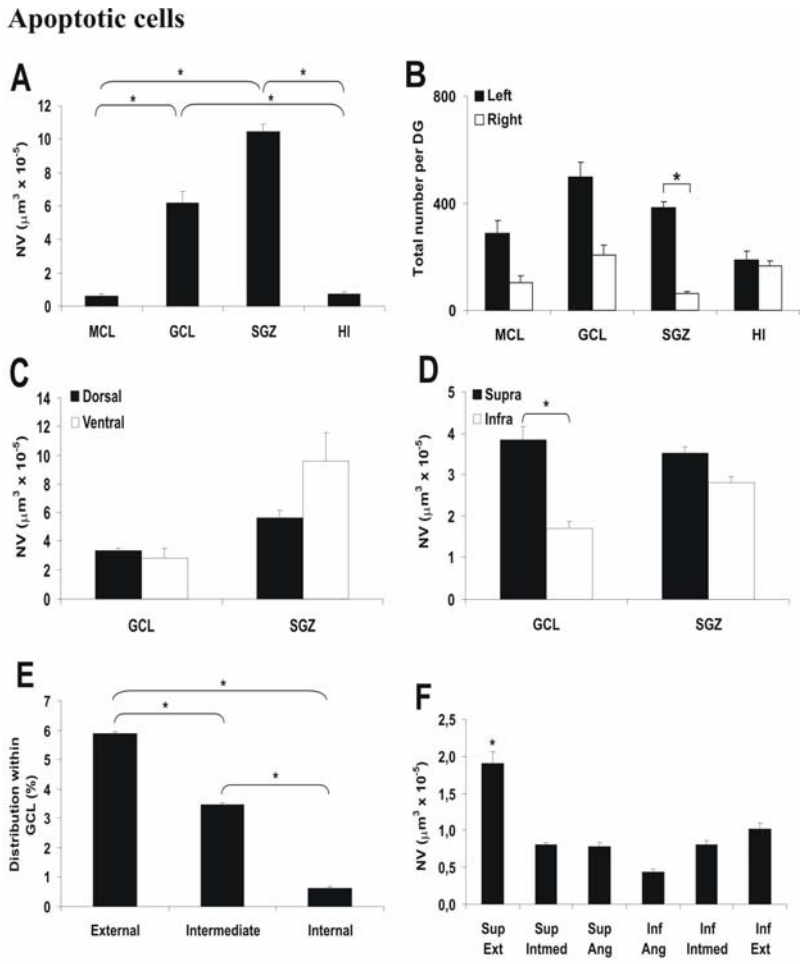


Figure 3

**Fig. 3 (A)** Graphic representation of the distribution of apoptotic cells in the dentate gyrus (DG) divisions: molecular cell layer (MCL), granular cell layer (GCL), subgranular zone (SGZ) and hilus (Hl). GCL vs MCL: p=0.002, GCL vs Hl: p=0.006; SGZ vs MCL: p≤ 0.0005; SGZ vs Hl: p≤ 0.0005. Mean CE=0.07. **(B)** Comparison between total number of apoptotic cells in left and right subdivisions of the dentate gyrus. SGZ: p≤ 0.0005. Mean CE=0.13. **(C)** Comparison of densities of apoptotic cells in dorsal vs ventral hippocampus. Mean CE=0.10. **(D)** Analysis of apoptotic cells density in granular supra- (Supra) and infra-pyramidal (Infra) cell layer in GCL and SGZ. GCL: p≤ 0.0005. Mean CE=0.07. **(E)** Analysis of apoptotic cell level in GCL longitudinal subdivisions. This comparison showed a gradient from internal to external layers and external layer as having the highest apoptotic levels. External vs Internal: p≤ 0.0005; External vs Intermediate: p≤ 0.0005; Intermediate vs Internal: p≤ 0.0005. **(F)** Comparison of apoptotic cells between transversal divisions in GCL and SGZ indicates superior external as having the highest levels of apoptosis. Sup Ext vs Sup Intmed: p=0.006; Sup Ext vs Sup Ang: p=0.0005; Sup Ext vs Inf Ang: p≤ 0.0005; Sup Ext vs Inf Intmed: p=0.006; Sup Ext vs Inf Ext: p=0.038. Mean CE=0.07]. * p≤ 0.005

**Annex II**

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CHAPTER 5.6

# Cellular and molecular analysis of stress-induced neurodegeneration – methodological considerations

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**Abstract:** Evidence that chronic hypercorticalism induces a broad spectrum of deleterious cellular effects in the brain has accumulated over the last two decades. These principal effects of hypercorticalism include neuronal atrophy, neuronal death and glial responses. Importantly, these changes, which may occur interdependently and/or concomitantly, lead to neurodegeneration. While there has been a significant expansion of the number of techniques available for examining effects of chronic stress in the brain, the cellular and molecular mechanisms underpinning stress-induced neurodegeneration are still only partially known. This article appraises the major current methodologies available for analyzing stress-induced neurodegeneration, and considers the advantages and limitations of each of these methods.

## What do we understand by stress?

Stress refers to the organism's attempt to mount an 'adaptive' (beneficial) response to aversive stimuli in order to maintain or restore homeostasis. Different sensory and motor systems are differentially activated depending on the quality and intensity of the stressful (aversive) stimulus, and the magnitude and duration of the response are influenced by the "context" of the stimulus (experience, mood, age, environmental factors) (Herman and Cullinan, 1997). Thus, extreme caution is necessary before generalizing about the effects of one particular "stress"; it is fair to say that much of the confusion existing in the field is a consequence of the false presumption that elevated corticosteroids mimic stress and/or one stressor is a representative of every stressor. Another important point to be noted is that prolonged elevations in corticosteroid secretion are a crucial accompaniment of the chronic response to stress (Sapolsky et al., 2000). Briefly, in the event that adequate adaptive mechanisms cannot be recruited, chronic stress will result in a state of chronic hypercorticalism and, as a consequence, deleterious effects, including immune suppression and a variety of mental disturbances will emerge (Sapolsky et al., 2000).

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## Forms of neurodegeneration

Numerous studies have demonstrated that chronic hypercorticalism induces a broad spectrum of deleterious cellular effects in the brain, which can be conveniently categorized as neuronal atrophy, neuronal death, and glial responses. These changes, which may occur interdependently and/or concomitantly constitute neurodegeneration. The neurodegenerative changes associated with hypercorticalism are by far less-marked than the damage seen in the so-called

neurodegenerative diseases (Parkinson's, Alzheimer's, etc.) and it would therefore, probably be more prudent to consider them as representative of the selective vulnerability of given brain regions. Most of the examples given in this review relate to the responses of hippocampal cells to stress (and pharmacological hypercorticalism). The hippocampus has been the most extensively studied brain region in this respect; its particular vulnerability to corticosteroids most probably reflects its high concentrations of corticosteroid (mineralocorticoid and glucocorticoid) receptors.

### *Neuronal atrophy*

An important notion to be kept in mind when referring to neuronal degeneration is that it does not necessarily imply the death of neurons. Indeed, most events perceived by a living organism (either positive or negative) are believed to modulate the structure of neuronal networks rather than lead to changes in neuronal number (Segal, 2002; Erickson et al., 2003).

To evaluate dendritic arborizations one can use the Golgi technique, which selectively impregnates single neurons with silver chromate (Camillo Golgi, 1843–1924). This method has provided indispensable information about the way in which sets of neuronal elements contribute to the gross structure of the neuropil and tracts. Impregnations show up as black, purple or reddish-brown against a pale yellow background; it is essentially a stochastic technique, the exact chemical mechanism of which remains unclear. This approach allows impregnated neurons and boundaries in any region of interest to be traced and reconstructed from successive serial sections. Two-dimensional (2D) analysis can be performed from traces obtained using a drawing tube attached to a light microscope. This type of analysis does not require any sophisticated equipment and has been widely used in the past. However, it has one major disadvantage: converting a 3D probe into a 2D probe results in a loss of information on the suppressed dimension. To achieve 3D reconstructions, cell bodies, dendritic arborizations, and boundaries of the region of interest should be drawn (under 25–100 × oil immersion objectives) and plotted in 3D using a video computer system (e.g., Neurolucida from MicroBrightField, Inc.). Three-dimensional models of neurons can be visualized using appropriate software. Three-dimensional reconstructions of neurons can be rotated around any of the *x*-, *y*-, and *z*-axes to allow the best visualization of the dendritic trees. Total dendritic lengths, number of segments/bifurcations, Sholl analysis (which provides an estimate of dendritic densities, based on the number of intersections between concentric circles centered in cell soma and the dendritic segments) and spine densities are just some of the parameters this analysis provides.

Importantly, the use of these techniques allowed the pioneers of neuroanatomy to recognize the organization of neuronal networks, and to eventually demonstrate the occurrence of remarkable alterations in dendritic trees and synaptic contacts following neuronal insults. Indeed, such knowledge existed long before the description of different forms of neuronal death.

Several studies in the 1990s demonstrated that hypercorticalism (pharmacological or stress-induced) induces alterations in cytoplasm organelles (Miller et al., 1989) and, ultimately, atrophy of CA3 pyramidal cell dendrites in the hippocampal formation; (Watanabe et al., 1992; Magarinos and McEwen 1995a); subsequent work confirmed these results in this neuronal population but also observed similar alterations in all the other major subdivisions of the hippocampal formation (Sousa et al., 2000). Furthermore, the later studies noted a marked loss of synapes in at least one of the links of the intrinsic hippocampal circuitry (the mossy fiber–CA3 connection). It may therefore be concluded that elevated corticosteroids trigger structural responses within cytoplasmic organelles, dendrites, axons, and their synaptic contacts; importantly, such changes do not necessarily involve the irreversible loss of neurons (Sousa et al., 2000).

Neuritic alterations of the type described above correlate with behavioral deficits and would appear to serve as the neuroanatomical basis of adaptive mechanisms underlying learning and memory (Erickson et al., 2003). The cellular basis of learning and memory has long been believed to include alterations in dendrites (mainly in spines) and in the number and structure of synapses (Cajal, 1893). The validity of this notion was explored in a number

of quantitative light and electron microscopic studies, which, in the majority of cases, showed that the richness of dendritic arborizations and the numerical density of synapses increases as a consequence of learning of novel behaviors. More recent studies have also shown that, despite numerical changes in dendritic spines and synapses, the cellular mechanisms of hippocampus-dependent associative learning include the remodeling of existing hippocampal synapses; these changes most likely reflect an involvement of signal transduction proteins and the transformation of silent postsynaptic synapses into active ones (Rusakov et al., 1997; Stewart et al., 2000). In light of these robust correlations between neuritic (dendritic spine and synapse) changes and cognitive performance, it seems warranted to conclude that perturbations of the former will result in impaired performance in hippocampus-dependent learning tasks.

Most interestingly, although the neuritic atrophy and synaptic loss referred to above would be expected to provoke some degree of functional impairment, together with the fact that these paradigms are not necessarily associated with neuronal cell loss, it seems more than likely that neuronal reorganization (regrowth of dendrites and axons and establishment of new synapses) of damaged neuronal circuits is an important mechanism allowing recovery from insults (McEwen, 1999). The above proposition appears to be valid insofar that studies in rats have shown that, whereas no significant structural reorganization occurs during or immediately after the termination of elevated corticosteroid levels (by pharmacological means or after the imposition of stressors), significant reorganization does occur within one month of withdrawal from the damaging stimulus (Sousa et al., 2000). This so-called "reactive synaptogenesis" occurs throughout the hippocampal formation and is commensurate with restoration of spatial learning and memory to levels found in control animals. Thus, the more recent findings match well with older observations that hypercorticalism-induced cognitive impairment is a reversible phenomenon.

Importantly, regeneration of dendritic, axonal, and synaptic elements does not seem to be compromised in conditions when profound neuronal loss has occurred, e.g., in the dentate granule cell layer after adrenalectomy, a manipulation accompanied by marked collapse of the mossy fiber inputs to the CA3 pyramidal layer. Administration of low doses of corticosterone to adrenalectomized rats can, at least partially, restore the total dendritic length of granule cells and the volume and surface area of the mossy fiber terminals (Sousa et al., 1999a). In addition, substitution therapy with corticosterone results in complete recovery of the volume of the suprapyramidal bundle, number, and surface area of mossy fiber–CA3 synapses, and the surface area of dendritic excrescences (Sousa et al., 1999a). These observations on the fine structural adjustments fit with results of other work showing that behavioral functions impaired by adrenalectomy can be partially reinstated by the administration of corticosterone (McCormick et al., 1997).

The evidence summarized above firmly indicates that alterations of the corticosteroid milieu can induce profound, but largely reversible, changes in the ultrastructural organization of the hippocampal formation; these bidirectional alterations, more than changes in neuron viability, may represent the neuroanatomical correlation of hippocampus-dependent learning and memory. Presently, there is no clear data available as to what neurochemical mechanisms might underlie the fine structural observations described above. However, NMDA and serotonin (5-HT) receptors appear to be key players since the administration of either NMDA antagonists or serotonin reuptake inhibitors have been shown to abrogate CA3 dendritic atrophy (Watanabe et al., 1992; Magarinos and McEwen, 1995b). Growth factors also seem to be likely mediators, as suggested by data showing that elevated corticosteroid levels (including those produced in response to stress) attenuate hippocampal brain-derived growth factor (BDNF) and nerve growth factor (NGF) levels (Smith et al., 1995; Hansson et al., 2000) and that adrenalectomy results in significant alterations in the levels of neurotrophin-3 and fibroblast growth factor-2 (FGF-2) (Barbany and Persson, 1992; Hansson et al., 2000). Finally, it seems highly plausible that neurotrophins play a major role in the neuritic regrowth seen after recovery from exposure to high corticosteroid levels because the recovery phase is characterized by an increase in their synthesis (Smith et al., 1995).

### *Neuronal death*

Neuronal death can occur through one of the two basic mechanisms – necrosis or apoptosis (see Fig. 1 and for review Majno and Joris, 1995). Necrosis is the unexpected death of cells resulting from "external damage," usually mediated via destruction of the integrity of plasma membrane and/or the trophic support of the cell. Morphologically, there is lysis of the plasma membrane of the swollen necrotic cell, which leads to release of cytoplasmic components into the surrounding tissue spaces. Inflammatory cells, attracted by the necrotic debris, trigger tissue destruction. Necrosis of isolated cells can occur, although necrosis usually affects large clusters. Consequently, there is significant tissue inflammation (with subsequent repair and scarring), with permanent alteration of architecture and function. Since necrosis usually ensues from cytotoxins, the process is completed rapidly within seconds-to-minutes.

Apoptosis differs from necrosis in that it involves the triggering of specific, sequentially occurring, events. Although the term apoptosis was originally coined to describe a specific morphological sequel, it is now known that apoptosis depends on activation of a genomic program; as such, the term apoptosis is frequently used synonymously with the term programmed cell death (Fig. 2). It should be mentioned that most current methods for the detection of apoptosis can only detect late stages of the process, and that some programmed cell death may not involve the mechanisms of apoptosis (e.g., oncosis – the term oncosis (derived from onkos, meaning swelling) was proposed in 1910 by von Recklinghausen precisely to mean cell death with swelling; oncosis leads to necrosis with karyolysis and stands in contrast to apoptosis, which leads to necrosis with karyorhexis and cell shrinkage). In contrast to necrosis, apoptosis is a much slower process; depending on the initiating stimulus, apoptosis requires from a few hours to several days for its complete manifestation. Conceptually, this form of cell death is analogous to "suicide," inasmuch as death results from the activation of the dying cell's own death machinery. Apoptosis, first recognized by embryologists, has now come to be recognized as being important for maintaining tissue homeostasis and to constitute a major component of many pathological responses, including neurodegenerative diseases. It is important to note that the genetic program for apoptosis can be triggered by both intrinsic (e.g., during histogenesis) and extrinsic factors, including stressful stimuli and exogenous corticosteroids, although the intracellular signaling cascades and morphological changes are essentially the same in both situations.

As already mentioned, apoptosis is now known to be important during embryogenesis/histogenesis but

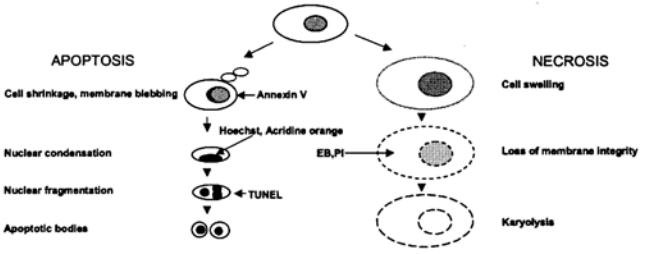


Fig. 1. Comparision of morphological changes in apoptosis and necrosis. Apoptosis, characterized by cell shrinkage, membrane blebbing, nuclear condensation, nuclear fragmentation, and apoptotic bodies developed in different stages of injury is shown on the left-side. As described in the main text, apoptotic cells can be identified in a variety of ways, some of which (annexin V-binding, Hoechst, acridine orange, and TUNEL staining) are indicated here. Note that the majority of methods are based primarily on changes in the properties of the cell membrane and nucleus. Necrosis, characterized by cell swelling, loss of membrane integrity, and karyolysis is shown on the right. Membrane-impermeable markers such as ethidium bromide (EB) and propidium iodide (PI) can be used to identify necrosis.

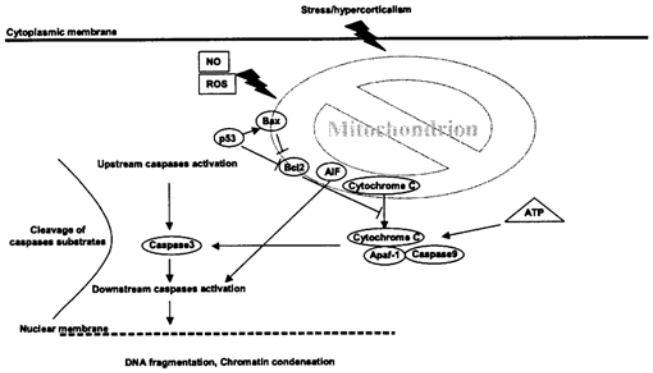


Fig. 2. Signal pathways in apoptosis. The mitochondrion as the integrator of apoptotic signals from stress or other factors such as nitric oxide (NO) or reactive oxygen species (ROS), can release cytochrome *c* and apoptosis-inducing factor (AIF); cytochrome *c*, together with Apaf1, Caspase9, and ATP activate caspase3, which, in turn, activates downstream caspases for DNA cleavage. Note that Bcl2 from the mitochondrial membrane can prevent mitochondrial pore formation, which antagonizes cytochrome *c* activity; Bax can increase mitochondrial permeability. Separation of living/apoptotic cells by flow cytometry. For flow cytometric analysis, ethanol-fixed cells are washed in phosphate–citrate buffer and stained with propidium iodide. As cells pass in front of a laser, they absorb, diffract, refract and reflect incident light, and emit fluorescence. The scattered light is focused by a lens into a photomultiplier, the emitted fluorescent signal is optically filtered through dichroic mirrors, and subsequently processed by wide bandpass filters selected to optimize the various fluorescent emissions; signals are detected by photomultiplier tubes, and based on fluorescence intensity profiles, living and apoptotic cells can be distinguished.

also in the course of normal tissue turnover. Of course, the mature brain is traditionally not regarded as an organ where cell and tissue turnover occurs, but with the increasing number of reports that, besides glial cells, neurons can also be generated in certain regions, the original concept does not seem to be strictly correct. Furthermore, it is being increasingly recognized that apoptosis makes a significant contribution to neural cell loss in pathological conditions, e.g., in neurodegenerative diseases such as Alzheimer's and Parkinson's disease (Honig and Rosenberg, 2000; Friedlander, 2003). It is also pertinent to mention that a revisionist view with respect to the distinctive roles and mechanisms of necrosis and apoptosis has emerged since the late 1990s; according to these authors it is now accepted that virtually any insult just below the threshold to induce necrosis results in an apoptotic response (McConkey, 1998). The cellular response becomes relevant in this process in that, in contrast to the situation in necrosis, apoptosis involves active processes within the dying cell and does not merely depend on the insult itself.

In contrast to most other cells, neurons have elaborate morphologies with complex neuritic arborizations that often extend long distances from the perikarya. It is in fact the richness of complex contacts between neurons that results in the establishment of functional networks. With this concept in mind, it is not difficult to accept that neuronal degeneration does not necessarily imply neuronal death; neuronal atrophy and synaptic loss also represent forms of nervous tissue degeneration. It was recently shown that the biochemical cascades leading to apoptosis can be activated locally in synapses and dendrites (Mattson, 2000), indicating a much more complex role for apoptosis than previously envisaged, i.e., in synaptic loss and dendritic remodeling.

### *Glial response*

Glia mediate neuroendocrine and neuroimmune functions that are altered in the face of a number of neuronal insults, including prolonged stress.

The biological functions of glia involve changes in shape, interactions with neurons and other glia, and gene expression. Glia cells become activated in the presence of ongoing neurodegeneration and progress to produce what is termed "reactive gliosis" (Nichols, 1999; Liu and Hong, 2003). Since good markers to distinguish normal from reactive glia are not commonly available, most researchers currently depend on well-defined morphological criteria. In several neurodegenerative conditions, astrocytes exhibit hypertrophy and signs of metabolic activation, and astrocytic processes begin to entwine neurons. Microglia also become activated and subsets of these cells increase in number and may enter the phagocytic or reactive stage. Glial markers of brain aging and glial activation include glial fibrillary acidic protein (GFAP) and transforming growth factor (TGF)-β1, which are increased in astrocytes and microglia, respectively (Nichols, 1999). Interestingly, steroids (Laping et al., 1994), such as those produced in the adrenals (Melcangi et al., 1997), regulate the interactions between glia and neurons, and glial gene expression, including GFAP and TGF-β1.

Despite the recognized relevance of the biological functions of glia, little is known about the effect of stress on hippocampal glial cells. Anecdotal evidence suggests an increase in glial cell number and signs of cytoplasmic transformation of astrocytes and microglia in areas of the brain implicated in stress-induced disorders (namely the prefrontal cortex and the hippocampus) (Ramos-Remus et al., 2002). Based on these findings, it appears that the hippocampal glial response to chronic stress may be similar to that found in endangered or challenged hippocampal environments, such as in ischemia.

A different line of evidence on the glial response to imbalances in the corticosteroid milieu has come from studies in surgically lesioned animals (Vijayan and Cotman, 1987). Animals with surgical entorhinal lesions concomitantly treated with hydrocortisone demonstrated more astrocytes and fewer nonastrocytes in the dentate outer molecular layer compared with untreated animals. Glia in the treated animals also showed a decrease in average optical density of cytoplasmic acid phosphatase staining. These findings suggest that hydrocortisone treatment prior to, and following, an entorhinal lesion accelerates lesion-induced migration of astrocytes to the outer molecular layer, and reduces the increase in microglial number resulting from the lesion. The observed effect on microglia may result from direct hormonal inhibition of local proliferation of microglia or from the well-known systemic anti-inflammatory action of glucocorticoids on monocytes, the putative precursors of brain microglia. In light of these findings it has been suggested that glucocorticoid hormones significantly alter the response of nonneuronal cells to neural tissue damage. Lending support to this view is the observation that adrenalectomized animals show induction of GFAP immunoreactivity, which occurs contemporaneously with neurodegeneration (Trejo et al., 1998). Although no variation in the total number of glial cells is found, signs of astroglial activation can be observed in the adrenalectomized group: astroglial cells change in size and shape, and their processes in the molecular layer, which normally show unipolarity become randomly organized (Sousa et al., 1997). Both effects are confined to the dentate gyrus and mossy fiber zone. The degeneration and astroglial reaction become more pronounced with increasing duration after adrenalectomy, and both can be prevented by placing animals on corticosterone replacement therapy. Results such as these illustrate the close relationship between the glial response and neuronal degeneration in the dentate gyrus following adrenalectomy, in terms of both, time and space (Sousa et al., 1997).

## What are the neural targets of stress-mediated degeneration?

Corticosteroids are secreted distal to their brain targets but distribution maps of their receptors serve as reliable indicators of their sites of action. In a landmark study on the rat brain, Reul and de Kloet (1985) reported that radioactively labeled corticosterone binds with differing affinities to two distinct receptors, and that the hippocampus showed the highest signal retention for both receptors; subsequent cloning studies revealed significant homologies between the high-affinity and low-affinity central and peripheral corticosteroid receptors: mineralocorticoid (MR) and glucocorticoid (GR) receptors, respectively. In vitro studies showed that the high-affinity binding site in brain can also bind aldosterone;

in practice however, the endogenous production of aldosterone only reaches concentrations sufficient to activate renal mineralocorticoid receptors (Funder, 1996); thus, cerebral MR show promiscuity in that, like GR, they bind corticosterone; however, since they have a ca. 10-fold greater affinity for corticosterone as compared to GR, MR are predominantly occupied during periods when corticosteroid levels are low, whereas GR only become occupied when corticosteroid secretion increases above a certain threshold (e.g., during stress or in pathological conditions). Further, the presence of two isoforms of the pre-receptor enzyme 11ß-hydroxysteroid dehydrogenase, involved in the interconversion of corticosteroids to active and inactive forms, contribute to the selective access to intracellular receptors (Yau and Seckl, 2001).

While GR are widely distributed throughout the brain, but are particularly concentrated in the hippocampus, hypothalamus, and lower brainstem, MR are almost exclusively confined to the hippocampus and other limbic structures such as the septum, central nucleus of the amygdala, the olfactory nucleus, and some hypothalamic nuclei (Van Eekelen et al., 1988; Ahima and Harlan, 1990; Ahima et al., 1991). Within the hippocampal formation, subfield-specific differences in MR and GR concentration profiles have been described: MR levels are high in CA1 pyramidal layer $\approx$ granule cell layer (dentate gyrus) > CA3 pyramidal layer, and GR are concentrated in the CA1 $\approx$ dentate gyrus $\gg$ CA3 (Van Eekelen et al., 1988). The functional significance, if any, of these differential patterns of receptor distribution may be inferred from the known functions of the particular brain nuclei displaying high levels of MR and GR expression and/or ligand binding. At this juncture, it is important to point out that while the described patterns of MR and GR occurrence in the various hippocampal subdivisions may serve as eventual predictors of function, they do not necessarily reflect the receptor repertoire of individual cells in any region; further, it is still not known to what extent receptor composition (concentration of individual receptors or co-localization of MR and GR in the same cell) determines the fate of a particular cell (e.g., survival vs. death) in response to changes in the corticosteroid milieu.

## Experimental paradigms for examining stress-mediated degeneration

Designing models of stress implies a clear definition of the question under study; more specifically, if one wants to determine the effect of stress upon a specific region of the brain, several issues need to be considered. One of them is adaptation; if a single stressor is applied for a prolonged period, then the organism tends to adapt to that stressor and the stress response gets blunted. A second issue to consider is unpredictability; even when applying different stressors, care must be taken to avoid adaptation, e.g., by applying a battery of stressors at different clock times and in random order. A final point to consider is that stressors vary in quality; for example, physical and psychological stressors activate different regions of the brain, with the former depending on perception by brain stem centers as opposed to the latter, which depends on the activation of higher regions of the brain (in particular the limbic system). Obviously, comparisons between different experimental paradigms (and the results therefrom) must also take into account factors such as intensity and duration/chronicity.

A commonly used approach in evaluating the cellular effects of stress involves decomposition of the effectors of these actions, e.g., by mimicking the endocrine response to stress by administering high doses of corticosteroids, a paradigm that does not exactly reproduce the physical, behavioral/emotional, and neurochemical manifestations of stress. Nevertheless, our current understanding of the actions mediated by the two corticosteroid receptors has largely benefited from the exploitation of the high selectivity of aldosterone (the prototypic MR agonist) and dexamethasone or RU28362 (prototypic GR agonists) as well as the antagonists spironolactone and RU28318 (for blocking MR effects) and RU38486 (for blocking GR effects). Further insights into the biological actions of MR and GR are now being gained from MR and GR gain- and loss-of-function mouse models (Muller et al., 2002). The use of such models has proved particularly useful in proving and understanding the importance and role of these receptors in stress-mediated neuronal damage, and neuronal disorders influenced by stress such as anxiety, depression, and dementia.

While in vivo models are necessary for the evaluation of stress effects, in vitro models are indispensable for understanding the cellular and molecular mechanisms underlying those effects. The latter approach is particularly amenable to analysis at the molecular level, but the major caveat here is that in vitro observations do not necessarily apply to the whole organism whose ultimate response to the same stimulus reflects an integration of a plethora of adaptive and signaling pathways emanating from cells with diverse properties, e.g., the liver can substantially influence the response of the brain to endogenous and exogenous stress hormones. As a result, a neurotoxic stimulus in vitro might just happen to be protective or to have no effect in the living organism.

## Use of stereology in analyzing neurodegeneration

Another extremely relevant issue to consider when designing an experiment is the sensitivity and specificity of the methodological procedures employed to test the hypothesis. Obviously, the analysis of stress-induced neurodegeneration also follows this rule. A common first analytical approach is to make observations on histological sections. Histological sections define the normal appearance of tissue and organs, and detect natural or induced alterations in structure. Histological descriptions often include terms such as "large," "small," "many," "few," "absent," or "present." Helpful as these terms are for the description of basic features, they are often open to subjectivity and, being qualitative, they do not allow statistical evaluation of the effects of a particular treatment, e.g., stress exposure. Quantitative data can take several forms, but all basically depend on counting cells in a section. One modern approach, superior to previous methods (Abercrombie, 1946; Weibel et al., 1966) is that of stereology, which is given detailed consideration below. Using stereology, one can obtain estimates of object volumes and derive numbers of objects from this data increasing the precision and relevance of data (Gundersen et al., 1988; West, 1999).

The principle behind stereology is to recreate or estimate the properties of geometrical objects in space. Its application to tissue or organ sections allows relatively precise estimation of the geometrical properties of the objects in a given section. As space has three dimensions, objects within it have properties for each possible number of dimensions, and objects within a given space can be defined in terms of their volumes (3 dimensions), surfaces (2 dimensions), lengths (1 dimension) and numbers. Each of these properties can be estimated by stereological methods, usually a two-step procedure involving: sampling and subsequently measuring.

A characteristic of many tissues and organs is that they contain a large number of the objects of interest, but too many to be measured individually. Producing a good sample is an essential step in stereological methods. Errors incurred during sampling can result in difficulties in obtaining meaningful stereological estimates later. Sampling usually starts before the investigator has any predictions as to the study outcome and even before the investigator has thought about applying stereological methods. To avoid later regrets, it is advisable to sample correctly from the very beginning; however, the researcher can be consoled by the fact that stereology-based sampling methods are compatible with all other types of analysis.

One usually wants to make statements about a structure (e.g., the hippocampus) or a cellular population (neurons) by sampling only a part of the structure or population. If such statements are to be valid for the entire structure or population, the sample must be a representative one. Selecting representative samples requires: (i) access to the entire structure or population; (ii) ability to recognize and/or define the entire structure or population; and (iii) that all parts of the structure or population contribute equally to the sample.

These pre-requisites can be met by random sampling in one of two ways: (i) Random independent samples – This is the most obvious approach in which one selects an initial location at random. After measuring the objects of interest, subsequent locations for measurement are chosen independent of the first. When a sufficient number of locations has been sampled, the individual measurements are averaged. Despite providing reliable and reproducible estimates, this method is, however, not an efficient sampling procedure. (ii) Uniform random systematic (URS) samples – In URS sampling, a random starting point is selected and samples are drawn at regular (or uniform systematic) intervals. Choosing

a random starting point means that all areas to be analyzed have an equal chance to contribute to the final sample measure. By eliminating sample clustering, the URS sampling procedure, on average, yields a more accurate estimate than the random independent sampling approach, and is the recommended method of choice. In practice, sections are selected using the URS sampling procedure at the time of tissue sectioning. If, for example, every fifth section is collected, the only requirement is to assure compliance with the need to randomly select the initial section in the series.

The next step in stereology is 'measuring' which involves relatively easy, routine work depending on identification of the object of interest and based on a simple set of rules. Curiously, the volume, surface, length, and number of objects are such basic parameters that it may be surprising to realize that methods for their accurate measurement only became available in the 1980s and did not enter widespread use until the 1990s. Stereological tools have now virtually replaced the earlier error-prone methods, which all suffered from the assumption that histological sections are two-dimensional images from which three-dimensional measurements were nevertheless attempted. The traditional methods involved certain well-grounded assumptions about the "missing dimension" in two-dimensional images. Inherently, the proximity to the true values achieved using such assumption-based methods, depended largely on how good the assumptions were.

In modern stereology, design-based methods have replaced assumption-based ones. These newer approaches involve measurements on a series of sections which in fact do have three dimensions. Therefore, information about the third dimension is based on fact, rather than assumption; obtaining precise measurements then depends on one other factor – the availability of good probes to apply to the sample. The selection of the adequate probe ultimately determines the precision of the estimation (West, 1999).

### *Estimation of volumes*

Estimating volumes using points is conceptually the easiest of all stereological methods and was first described by the Italian mathematician Bonaventura Cavalieri (1598–1647). The "Cavalieri Principle" holds that, if one places a grid of regularly spaced points over an object of interest, the measured surface area will be a function of the number of points falling within it. To calculate the volume of an object (in our case, section), one simply has to multiply the average areas of different sections by the thickness of each section. The point-counting principle can, theoretically, also be applied to very small objects like cells, but this would require very thin sections in order to reduce error and the ability to identify the object in consecutive sections. Other approaches, like the nucleator – in which a point associated with a small particle (e.g., a nucleolus within a cell) is identified and from which rays are extended until the intersection of particle's boundaries to allow the estimation of its profile area and, subsequently, the absolute volume of the particle – have been developed for this purpose (Gundersen et al., 1988).

### *Estimation of total cell numbers*

Measuring neuronal loss has preoccupied many neuroscientists interested in the effects of stress and glucocorticoids on the brain, in particular, the hippocampus. Such information can be generated by simply counting the number of cells in a given section and comparing the values obtained with those for sections from an anatomically matched area in control (e.g., nonstressed) subjects. This procedure yields a probable estimate of cell number per unit area ($N_A$); to date, this is probably the most widelyused method to count neurons. The precision (relevance) of such an estimate relies entirely on how similar the sections being compared are. A serious (but common) error of such estimates is the "reference trap," which refers to how variation in the volume of reference can affect the final result. This can be illustrated by considering the fact that because the $N_A$ of granule cells in the hippocampus in two different sections from different experimental groups is similar, it does not necessarily follow that the total number of cells in each section is the same; this is because the volume (derived from the third dimension, which is not taken into account in deriving the $N_A$ value) of the dentate granule cell

layer might differ significantly between individual sections and subjects. Design-based methods (e.g., estimating neurons using volumes within a probe) help avoid the introduction of such biases (West, 1999).

Essentially, estimating numbers within a volume is just the corollary of estimating volumes with points. One takes two adjacent sections that are thinner than the diameter of the object (e.g., nuclei) to be counted; the objects visible in the second, but not first, section are counted. Then, the number of objects in the volume represented by the two sections will, on average, correspond to the number of objects counted in the second. This approach is called the (physical) dissector because the counting principle is based on a comparison of two sections. Application of this technique provides the numerical density ($N_v$) of objects (e.g., neurons) within a region of interest. Now, if the total volume of the structure (e.g., hippocampus) is known, the total number of objects (e.g., neurons) can be derived from the product of $N_v$ and the total volume.

The optical fractionator is another means for obtaining the total number of objects in a given 3D structure. It is based on the combination of systematic sampling (which yields an estimate of the fraction of the tissue – fractionator) and the dissector in thick optical sections that intrinsically have three dimensions.

## Detection of cell death

The application of stereological methods to histological sections (e.g., stained with Nissl, Giemsa) can certainly provide information of neuronal loss based on the comparison of total number of surviving neurons between experimental groups (West et al., 1991; Sousa et al., 1999b). However, this approach can also be applied in combination with markers of neuronal death to directly determine the number of dying cells at a particular time-point. Several specific staining methods for detecting neurodegeneration have been developed but their use has not yet been generalized. The earliest markers of neuronal degeneration were based on silver-impregnation methods that provide unspecific indications of degeneration in neuronal soma and neurites.

More recently, the use of two anionic fluorescein derivatives have proved very useful for the simple and definitive localization of neuronal degeneration in brain tissue sections. Initial work on the first generation fluorochrome, Fluoro-Jade, demonstrated the utility of this compound for the detection of neuronal degeneration induced by a variety of well-characterized neurotoxicants, including kainic acid, 3-nitropropionic acid, isoniazid, ibogaine, domoic acid, and high doses of dizocilpine maleate (MK-801) (Schmued et al., 1997). After validation, the tracer was used to reveal previously unreported sites of neuronal degeneration associated with other neurotoxicants. Preliminary findings with a second-generation fluorescein derivative, Fluoro-Jade B, suggest that this tracer is a specific and selective marker for the identification of neurons undergoing degeneration (both apoptotic and necrotic) (Eyupoglu et al., 2003); Fluoro-Jade B also provides improved staining and can stain the distal portion as well as the proximal portion of the dissected axon (the so-called anterograde and retrograde degeneration after axotomy). Furthermore, Fluoro-Jade tracers can be combined with other histologic methods, including immunofluoresence that can help in discriminating different types of neurodegeneration to obtain information on the neurochemical identity of the affected cells (Schmued and Hopkins, 2000); recent preliminary findings on a number of specialized applications of Fluoro-Jade include the detection of apoptosis, amyloid plaques, astrocytes, and dead cells in tissue culture.

An early observation concerning apoptosis was that cells entering apoptosis showed dramatic and characteristic changes in nuclear shape and organization (Fig. 1) (see for review Kerr et al., 1972; Wyllie, 1980; Ucker, 1991). It is still probably correct to say that the characteristic change in nuclear morphology is the most accurate indicator of the involvement of apoptosis in the death of a cell. This is true even in light of the apparently paradoxical observation that nuclear fragmentation per se is not essential for apoptosis; enucleated cells can still undergo other changes characteristic of apoptosis. This unequivocally demonstrates that the effectors of the apoptotic machinery are located in the cytoplasm. However, under normal conditions, changes in nuclear morphology remain an early and relatively unequivocal

hallmark of apoptosis, with such changes occurring at an early point in the series of morphological events, usually soon after the onset of surface blebbing.

Apoptosis is an ATP (energy)-dependent process (Reed and Green, 2002). Since ATP levels fall to a point where the cell can no longer perform basic metabolic functions, the cell will die. Apoptotic cells exhibit significant reductions in their ATP levels, which can serve as an early marker of cell death. Depletion of energy pools is, however, not specific to apoptosis. Either exposure to toxic agents (secondary necrosis) or metabolic damage (primary necrosis) can also induce drops in ATP levels, albeit rapid ones (Leist et al., 1999), followed by necrotic cell death. The change in both ATP and ADP levels (ADP/ATP ratio) has been used to differentiate apoptosis from necrosis (Bradbury et al., 2000). In contrast, cell proliferation and growth arrest can both be recognized by increased levels of ATP and decreased levels of ADP. Determination of the ADP/ATP ratio offers highly consistent results and with excellent correlation to other markers of apoptosis (e.g., TUNEL-based techniques and caspase assays) (Bradbury et al., 2000).

c-Jun N-terminal kinase (JNK) is one of the main MAP kinase groups identified in mammals. Recent evidence suggests that activation of JNK plays an important role in neuronal apoptosis and other physiological and pathological processes (Ham et al., 2000). For measuring JNK activity easily in a large number of samples, one can use an assay that utilizes an N-terminal c-Jun fusion protein bead to selectively "pull down" JNK from cell lysate; c-Jun phosphorylation is then measured using a phospho-c-Jun-specific antibody. Alternatively, one might analyze JNK-specific activity by determining the phosphorylation of c-Jun by Western blotting using a phospho-c-Jun-specific antibody. Given the involvement of JNK in signaling pathways, which may not be directly related to apoptosis, care needs to be exercised in interpreting results obtained with such methods.

One of the first questions to resolve whenever searching for neurodegeneration, whether necrotic or apoptotic, is the ability to distinguish if the cells undergoing degeneration are neurons or glial cells. For this, immunohistochemistry is the most convenient and commonly used approach. Using specific antibodies for each cell population, one can easily identify the lineage of dying cells. Numerous neural cell type-specific (neurons, astroglia, oligodendrocytes, etc.) markers are currently available. For example, one may use antiGFAP to label astrocytes, antidoublecortin to identify neuroblasts (stem cells), antiNeuN to mark mature, differentiated neurons, or antiTuJ1 to study fibers.

As mentioned already, apoptosis is a genetically programmed phenomenon. A complex network of genes (Steller, 1995; Lossi and Merighi, 2003), in particular encoding members of the Bcl-2 family of proteins, play a central role in the regulation of apoptosis. Here, we focus on Bcl-2 family members as these have received most attention in the context of this article. The Bcl-2 family of proteins comprises death-inducer (proapoptotic) molecules such as Bax and Bcl-$x_S$ and death-repressor (antiapoptotic) molecules such as Bcl-2 and Bcl-$x_L$. These various proteins, which can homo- or heterodimerize with each other, are activated by physiological or injurious stimuli, and appear to operate upstream of events leading to the final execution phase of the apoptotic process; the latter results from the activation of cysteine proteases, the caspases.

Caspases convey the apoptotic signal in a proteolytic cascade, with caspases cleaving and activating other caspases that then degrade other cellular targets that lead to cell death (Friedlander, 2003). Caspase activation can directly initiate the permeability transition of the mitochondrial membrane, resulting in the release of several mitochondrial proteins (see Fig. 2 for a simplified scheme). The large number of products developed to study caspases and their substrates is indirect testimony to their importance; because of space constraints, the authors here only review a few of these. Caspase-3 is a key protease that becomes activated during the early stages of apoptosis. Synthesized as an inactive proenzyme, the activated form cleaves and activates other caspases, in addition to cleaving specific targets in the cytoplasm and nucleus (e.g., DNA and nuclear membrane fragmentation). Once activated, caspase-3 serves as a marker for cells undergoing apoptosis. Several biotin- or FITC-tagged anti-active caspase 3 antibodies are available, facilitating their routine use (Gown and Willingham, 2002). Because caspase

activity is likely to be the most specific indicator of the apoptotic process, the assay of caspase activity through the detection of specific cleavage products in target proteins represents a theoretically valid approach for measuring apoptosis. Recently, antibodies to the caspase-generated cleavage products of cytokeratin 18 have appeared on the market, with several studies demonstrating their utility, especially in cell culture, but probably also in fixed tissue sections (Leers et al., 1999). However, cytokeratin 18 is expressed only in certain cell types and this antibody is not broadly applicable to all cell types. The use of antibodies specific for more generally distributed cleaved substrates of caspases, such as the cleaved form of caspase 3, would have more general applicability (Srinivasan et al., 1998). Owing to their cell-permeable nature, a new line of cell-permeable fluorogenic caspase substrates enables the visualization of intracellular protease activities by standard fluorescence microscopy or multiparameter flow cytometry (see below) in living cells. The substrates, designed for caspase-1, caspase-6, caspase-8 (the caspase-3 processing enzyme), and caspase-9, detect early events in the apoptotic pathway before DNA degradation has started (Davis et al., 1998; Komoriya et al., 2000). Recently, these caspase substrates have been used to demonstrate that the pattern of caspase activation is not only dependent on the apoptosis-inducing agent employed, but also on the cell type (Komoriya et al., 2000). Events occurring downstream of caspase-3 activation include cleavage of poly(ADP-ribose) polymerase (PARP), an enzyme implicated in DNA damage and repair mechanisms. Cleavage of PARP from the native 116 kDa to 85 kDa is considered a hallmark of apoptosis (Sallmann et al., 1997). The availability of FITC-tagged anti-PARP antibodies therefore, provide another useful marker of apoptosis.

In healthy cells, cytochrome *c* is located in the space between the inner and outer mitochondrial membranes. An apoptotic stimulus triggers the release of cytochrome *c* from the mitochondria into the cytosol where it binds to Apaf-1. The cytochrome *c*/Apaf-1 complex activates caspase-9, which then activates caspase-3 and other downstream caspases. Cytochrome *c* released from the mitochondria into the cytosol can be detected by Western blotting using antibodies directed against cytochrome *c*. The procedure is simple, straightforward, and provides an effective means for detecting cytochrome *c* translocation from mitochondria into cytosol during apoptosis (Jemmerson et al., 1999).

As already alluded to, Bcl-2 family proteins form complexes, these complexes can enter the mitochondrial membrane where they regulate the release of cytochrome *c* and other proteins. When Bax, for example, localizes to the mitochondrial membrane, it acts to increase mitochondrial permeability, induces the release of cytochrome *c* and other mitochondrial proteins, leading to apoptosis ultimately. In contrast, Bcl-2 and Bcl-$x_L$ prevent mitochondrial pore formation and therefore, block apoptosis. Antibodies (applications include immunocytochemistry, Western blotting) and gene probes (for Northern blotting, in situ hybridization histochemistry, and polymerase chain reaction analysis) are now available for measuring most key members of the Bcl-2 family in a variety of species, including humans, rats, and mice. Such studies have shown that Bcl-2 levels in the brain decline rapidly after birth, except for those areas displaying postnatal neurogenesis such as the dentate gyrus of the hippocampus. Further, numerous studies have shown that Bcl-2 expression can be induced in the adult brain, including the hippocampus, upon experience of various noxious stimuli. Unlike that of Bcl-2, the expression of Bcl-$X_L$ occurs in neurons from early development through to senescence. The proapoptotic protein Bax is expressed through all life stages whereas the smaller proapoptotic splice variant of Bcl-2, Bcl-$X_S$ is only barely detectable in the mature brain. To date, there is no evidence that corticosteroids, which represent the endocrine response to stress, can directly regulate or interact with any members of the *bcl-2* gene family. Rather, corticosteroids appear to influence the pro- and antiapoptotic gene expression and activity by interacting with p53, a ubiquitously distributed tumor suppressor protein, which has been shown to induce and repress the transcription of *bax* and *bcl-2*; glucocorticoids were recently shown to enhance the transactivation potential of p53 (Crochemore et al., 2002). Although measurements of gene or protein expression of Bcl-2 family members might be reasonably expected to correlate with apoptosis, recent studies have shown that absolute levels of these molecules do not reflect the actual viability of

neurons in situ. Rather, the ratio of expression of pro-apoptotic (e.g., Bax) to antiapoptotic (e.g., Bcl-2) molecules factor has proven to be the factor determining neuronal survival (Almeida et al., 2000) insofar that this derivative correlates with the incidence of apoptosis measured by histochemical techniques such as TUNEL (see below).

Disruption of the mitochondrial transmembrane potential is one of the earliest events after apoptosis induction. Normally, cellular energy generated by mitochondrial respiration accumulates in the transmembrane space as an electron gradient called the mitochondrial transmembrane potential $\Delta\Psi_m$. Disruption of the mitochondrial transmembrane potential occurs following the onset of apoptosis. Using a fluorescent lipophilic cation as a mitochondrial activity marker, one can measure differences in the fluorescence displayed by healthy cells versus apoptotic cells: in healthy cells, the dye accumulates and aggregates in the mitochondria, producing a bright red fluorescence, while in apoptotic cells the fluorescence cation cannot aggregate in the mitochondria because of the altered transmembrane potential, thus remaining in its monomeric (green fluorescent) form within the cytoplasm. These fluorescent signals are analyzed by flow cytometry using the FITC channel for green monomers and the propidium iodide (PI) channel for red aggregates. Additionally, apoptotic and healthy cells can be viewed simultaneously by fluorescence microscopy using a wide-band pass filter. Some kits combine detection of disrupted mitochondrial transmembrane potential with changes in the composition of the plasma membrane.

Flow cytometry can be summarized as a method for measuring physical and biochemical features of cell components on a cell-by-cell basis, primarily by optical means. Fluorescent dyes or fluorophore-conjugated antibodies are used to report the quantities of specific cellular components, density of cellular markers and receptors – or even activation state of various enzymes. Put simpler, flow cytometers are highly sophisticated fluorescence microscopes, where fixed or living cells are not attached to a well-defined surface, but rather travel one by one, by continuous flow of a stream of the suspension past a sensor. Each cell scatters some of the excitation laser light, and the labeled cells emit fluorescent signals from the dye. These two parameters are sensed by photodetectors, data are collected, and processed by a computer.

The term 'FACS' is Becton-Dickinson's registered trademark and is an acronym for "Fluorescence-Activated Cell Sorter." FACS is therefore, a machine that can rapidly separate cells in a suspension, based on the size and color of their fluorescence. (Note: not all flow cytometers are necessarily able to separate cells into different vials, but all can analyze the distribution of cell size and/or physical or biochemical cellular properties). These particular features of flow cytometric methods allow the identification and quantification of apoptotic cells as well as possible mechanisms of cell death.

The main flow cytometric approaches that can be used to identify apoptotic cells may be summarized as follows: (1) Apoptosis-associated changes in cell size and granularity can be detected by analysis of laser light scattered by the cell. (2) Using annexin V in combination with propidium iodide (PI), it is possible to differentiate between healthy, early apoptotic, and necrotic cells on the basis of the distribution of plasma membrane phospholipids as well as changes in membrane integrity. (3) Fluorochromes like Rhodamine 123 (Rhod123) or 3,3′-dihexiloxadicarbocyanine ($DiDOC_6$) reveal decreases in the mitochondrial transmembrane potential ($\Delta\Psi_m$) that occurs early during apoptosis. (4) Apoptotic cells can be recognized by their fractional DNA content, or by the presence of DNA strand breaks using fluorochrome-labeled nucleotides attached to the 3′-OH termini in a reaction catalyzed by exogenous terminal deoxynucleotidyl transferase (TdT). As regards the identification of putative mechanisms of cell death, after labeling with primary and fluorescent secondary antibodies, one can detect and measure: (a) cellular levels of death-related proteins (members of Bcl-2 family, proto-oncogenes like c-myc and ras, tumor-suppressor genes such as p53, etc.) or (b) study particular cell functions, such as mitochondrial metabolism, in the context of cell sensitivity to apoptosis.

The main virtue of flow cytometry lies in the possibility of multiparametric, correlated analysis of a multitude of cell attributes and markers, thus addressing problems of cellular heterogeneity. Flow cytometry also provides more effective data acquisition as compared to fluorescence microscopy

(which has similar capabilities, but in which the sample size is limited up to a few hundred cells. Flow cytometry can easily measure 10,000–100,000 cells per sample!). There are, however, certain difficulties associated with this technique, which have to be taken into consideration when using flow cytometry in general. With respect to cell death detection, a major problem is that the single parameter on which the identification of apoptotic or necrotic cells relies on, may be absent, when apoptosis is atypical. Moreover, in the case of nonfixed, living cells, the dissociation procedure may damage the plasma membrane, resulting in PI (a widely used cell viability dye) to enter and label the cells as if they were dead. Clumping of cells may also pose technical difficulties. Since high-speed FACS machines use high pressure to achieve rapid acquisition rate, limitations such as cell type and viability must also be considered.

Externalization of phosphatidylserine (PS) and phosphatidylethanolamine are hallmarks of changes in the cell surface during apoptosis. Annexin V binds to PS with strong avidity and can be used as a marker of PS externalization using either microscopy or flow cytometry (when fluorescent-labeled annexin V is applied). Importantly, annexin V-binding cannot be applied to tissue sections or adherent cells, and when flow cytometric analysis is used on cell suspensions. PS are phospholipids only present on the cytoplasmic face of the plasma membrane and other internal membranes, and it remains unclear as to why certain subpopulations of cells ($< 30\%$) entering apoptosis externalize PS at a very early stage of the process, just after the fragmentation of the nucleus begins. However, since inhibition of caspase activity blocks PS externalization, a role for caspases is indicated. It is important to keep in mind that, during necrosis or the terminal lytic steps of apoptosis, PS that are actually localized on the inner face of the membrane might be accessed by annexin V, giving rise to false positives. When combined with a vital dye such as the red fluorescent DNA-binding compound PI, FITC-annexin V labeling can be used to distinguish necrotic from apoptotic cells; this is because PI does not penetrate live cell membranes or cells in the early phases of apoptosis but only cells that have lost membrane integrity as a result of necrosis or very late apoptosis.

The permeability of the plasma membrane is substantially different in necrotic and apoptotic cells, a fact that can be taken advantage of in the distinction between these forms of cell death. Thus, it is possible to distinguish between stages of apoptosis mainly on the basis of the plasma membrane permeability changes. Large-molecular weight DNA-binding dyes, such as PI or the homodimer of ethidium bromide (EB), cannot enter intact cells because of their large size and, without permeabilization treatments, do not label apoptotic cells until the final stage of cell lysis. On the other hand, in ethanol-fixed cells, which have been subsequently washed in phosphate–citrate buffer, extraction of the low-molecular weight DNA from apoptotic cells takes place, and apoptotic cells appear to the left of the normal G1 peak after PI staining (Fig. 3). This is a fast, simple, but not very specific, method for detecting apoptosis, and has the disadvantage that apoptosis of cells in late S phase or from G2 may be missed. Smaller dyes that can attach to DNA (such as DAPI, Hoechst 33342 or 33258), are furthermore able to enter, and differentially label apoptotic and healthy cells based on the condensation and subsequent fragmentation of the chromatin, which occurs early during apoptosis. Using flow cytometry, for instance, one can distinguish between healthy, apoptotic and dead cells by the simultaneous use of the blue-fluorescent Hoechst 33342 dye (which stains the condensed chromatin of apoptotic cells more brightly than that of normal cells) and PI, which labels dead cells (Pollack and Ciancio, 1991). Acridine orange (AO) (another cell-permeant nucleic acid-binding dye that emits green fluorescence when bound to double-stranded DNA, and red fluorescence when bound to single-stranded DNA or RNA) is another useful probe for identifying apoptotic cells, because its metachromatic fluorescence is sensitive to DNA conformation. Careful combination of fluorescent dyes, furthermore, allows even more accurate determination of different stages of the apoptotic process: for example, 7-aminoactinomycin D (7-AAD) can be used alone or in combination with Hoechst 33342 to separate populations of live, early apoptotic, and late apoptotic, cells (Schmid et al., 1994). In mixed cell populations, however, identification of cell types is necessary, and has also to be taken into consideration. For instance,

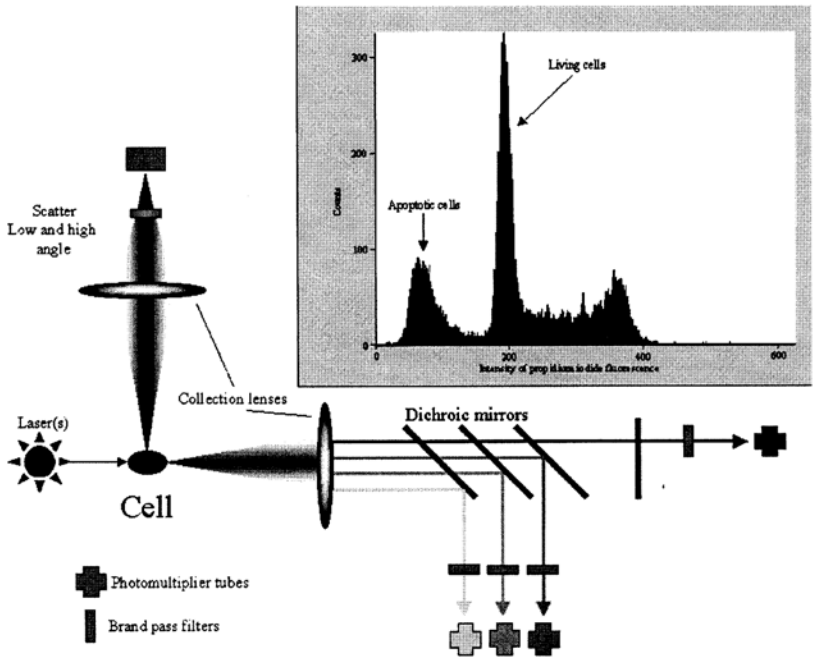


Fig. 3. Separation of living/apoptotic cells by flow cytometry. For flow cytometric analysis, ethanol-fixed cells are washed in phosphate–citrate buffer and stained with propidium iodide. As cells pass in front of a laser, they absorb, diffract, refract, and reflect incident light, and emit fluorescence. The scattered light is focused by a lens into a photomultiplier, the emitted fluorescent signal is optically filtered through dichroic mirrors, and subsequently processed by wide-band pass filters selected to optimize the various fluorescent emissions; signals are detected by photomultiplier tubes, and based on fluorescence intensity profiles, living and apoptotic cells can be distinguished.

the combination of acridine orange and ethidium bromide (AO/EB) is useful to accurately differentiate between healthy, early apoptotic, late apoptotic, and necrotic cells (Liegler et al., 1995), but cannot be used for phenotypic analyses due to the broad emission spectrum of AO and EB. Certain techniques, like DNA strand-break labeling by terminal deoxynucleotidyl transferase (TdT) can overcome this problem, but are technically very demanding. TdT adds biotinylated or digoxygenin-labeled nucleotides to the strand-breaks in the DNA of apoptotic cells – apoptotic cells therefore, can be detected by using fluorochrome-labeled streptavidin conjugates or fluorochrome-labeled anti-digoxygenin antibodies in flow cytometric analysis. By combining this procedure with phenotypic markers tagged to other dyes, it is even possible to obtain cell cycle profiles in cells of a given phenotype (Li et al., 1996).

As mentioned earlier, changes in plasma membrane permeability are signs of late phases of cell lysis. Changes in mitochondrial membrane permeability, however, occur much earlier during apoptosis, and are considered to be a distinctive feature of early programmed cell death. The mitochondrial permeability transition (MPT) is intimately linked to the opening of a "megachannel," the permeability transition pore (PTP). Ionic equilibration through the PTP results in disruption of the mitochondrial

transmembrane potential ($\Delta\Psi_m$), uncoupling of the respiratory chain, and release of cytochrome *c* into the cytoplasm. Of all these features, changes in the mitochondrial permeability can be relatively easily followed by application of fluorescent dyes (e.g., $DiOC_6$), while the subsequent ionic and electrical fluctuations can be investigated by patch-clamp techniques or certain fluorophores. While certain drugs, like the green-fluorescent calcein (which is produced from the nonfluorescent calcein-AM form within the cell itself) are used to indicate PTP opening, and, subsequently, the taking up of the dye into the mitochondrial matrix, others (like JC-1, JC-9, or $DiOC_6$) do not just simply accumulate in the mitochondria, but also indicate changes in $\Delta\Psi_m$ in single-cell imaging or flow cytometric assays. Other dyes, like MitoTracker® Red CMXRos can be fixed by aldehyde-based fixatives and can thus be used for other subsequent analytical procedures such as immunocytochemistry, DNA end-labeling, in situ hybridization, or counterstaining. Ionic concentrations in the mitochondria can be monitored using patch-clamp techniques or fluorescent dyes like the $Ca^{2+}$-sensor Rhod-2.

Loss of DNA integrity is characteristic of apoptosis (Collins et al., 1997). When DNA extracted from apoptotic cells is analyzed using gel electrophoresis, a characteristic internucleosomal "ladder" of DNA fragments (typically, 180–200 bp in length) is revealed (Compton and Cidlowski, 1986; Walker et al., 1999); larger DNA fragments have also been seen at earlier stages in apoptotic cell cultures. Although these electrophoretic methods are commonly used in apoptosis detection, the results they provide can present interpretational difficulties. Also, these methods cannot be easily applied, requiring extraction of DNA from large numbers of cells undergoing apoptosis in a relatively synchronous way; however, such synchrony is not always present, especially in tissues (Collins et al., 1997), and as noted above, apoptosis is a relatively rare event, occurring in only a subset of cells within a given structure, thus raising problems of sensitivity.

A widely used method that has contributed much to our knowledge of stress- and corticosteroid-induced apoptosis in the brain is also based on the detection of DNA strand-breaks. This approach detects 3′-OH ends of single-stranded DNA after the addition of labeled nucleotides to the open ends of DNA in a procedure known as in situ end-labeling (ISEL). The latter may be achieved using either *E. coli* polymerase (or its Klenow fragment) in a method called in situ nick-translation (ISNT), or terminal transferase in a method referred to as terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) (Modak and Bollum, 1972; Gavrieli et al., 1992; Jin et al., 1999). These methods allow the cytochemical demonstration of free DNA strand openings.

TUNEL staining is now widely used for the detection of apoptotic cells in tissue sections and cells in culture. Despite its apparent simplicity, unless used optimally, this technique may lack sensitivity and, worse, specificity. For example, TUNEL can reportedly label both apoptotic and necrotic cells, and potentially, proliferating cells also, although these problems are less-frequently encountered in tissue sections than when cultured cells are stained. Moreover, as already noted in the main text, apoptotic cells can be easily recognized on the basis of their unambiguous morphological characteristics. With regard to mitotic cells, it deserves mentioning that although chromatin condensation at telophase may mimic apoptosis, the greatest analogy between mitotic and apoptotic aspects occurs in abortive mitosis, a form of cell division that leads to active cell death (sometimes named "mitotic catastrophe").

The major problems associated with the TUNEL technique, especially in tissues, can be summarized as follows: (i) without pretreatment, TUNEL sensitivity is poor and can lead to false negatives; (ii) established pretreatments (proteinase K, microwaves) can easily result in labeling of morphologically normal nuclei; and (iii) the method depends on good fixation and can prove problematic when large tissue blocks are used (outside–inside gradients of penetration of fixative). Other considerations include: (i) the DNA breaks, which are targeted by TUNEL, are less accessible than intact DNA; (ii) besides apoptosis, DNA recombination, replication, repair or compaction–relaxation during mitosis, tissue electrocoagulation, autolysis, fixation, paraffin embedding, cutting, and pretreatments with H2O2, detergent, proteinase K, and microwaves can all result in DNA breaks; and (iii) DNA compaction

(a hallmark of apoptosis) and protein cross-linking and precipitation induced by fixation can mask the 3-OH recessed ends.

Despite the above caveats, TUNEL is still regarded as a reliable marker of the DNA fragmentation, which typically occurs in apoptosis. The key to distinguishing between apoptotic and nonapoptotic DNA is the cautious use of "break disclosure" reagents (detergents, proteases, microwaves). Extensive tests have led some authors to propose that optimal staining results from qualitative adaptations of retrieval techniques rather than retrieval reinforcement; for example, quite different pHs are necessary to obtain specific labeling in formalin- versus Bouin-fixed tissues. When fixation is controlled (e.g., homogeneous and light) as is usually the case in prospective studies, proteinase K alone may be sufficient for all cross-linking aldehyde fixatives (paraformaldehyde, formalin, B5). Proteinase K and microwave treatment may be necessary when tissues are fixed for too long and/or in precipitating solutions (Bouin's).

Nonspecific (background) staining can also present a problem, even when optimal pretreatments are applied. This can be overcome by optimizing the detection system, e.g., dilution of the enzyme-coupled antibody, choice of enzyme, careful monitoring of color development. Absence of standardization of color reaction implies suboptimal quantification of those cells which might otherwise show morphological signs of apoptosis. Also to be remembered is that all labeled cells, irrespective of intensity of labeling, should to be counted as long as they show morphological features of apoptosis.

Another method for detecting these single-strand ends is the use of monoclonal antibody reactive with single-stranded DNA (Naruse et al., 1994; Frankfurt et al., 1996). Since preservation/fixation procedures can have dramatic effects on the detection of single-stranded DNA (Labat-Moleur et al., 1998; Tateyama et al., 1998), careful consideration must be given to this issue and optimized for each cell type or tissue. The investigator should also keep in mind that in cases of overfixation, for example, open DNA strands will be inaccessible to assay reagents (Nakamura et al., 1997). This can be overcome by introducing protease treatments prior to ISNT or TUNEL procedures. Proteases must be used cautiously – protease treatments can mimic the actions of endogenous caspases, thus leading to artefactual DNA strand-breaks. Here, it is also important to note that, depending on permeabilization and fixation protocols, some methods detect so-called preapoptotic nuclei in which strand breaks are detected in the absence of apoptosis-like changes in the morphology of the nucleus. Alternatively, positively labeled strand-breaks may not correlate with nuclear fragmentation in individual cells, or DNA strand-breaks may only become detectable at relatively late stages of the apoptotic process (Collins et al., 1997). Recently, a number of authors have indicated reservations about the use of the TUNEL and ISNT assays for detecting apoptosis. It has become apparent that single-stranded DNA ends are not necessarily specific for apoptosis since they may also occur in necrotic cells (Kockx et al., 1998; Mizoguchi et al., 1998). Therefore, although these methods have been, and remain, very useful (their major advantage being that they can be applied directly to intact tissue sections, thus providing good anatomical resolution), the results they yield must be treated with extreme care; for example, in our studies (e.g. Hassan et al., 1996), we only consider TUNEL-positive cells as apoptotic if they simultaneously display the typical morphological characteristics of apoptotic cells; positively stained cells, which have a clearly defined nucleus and cell body are

Table 1. Dyes commonly used for quantifying apoptosis by flow cytometry

| Marker dye | MW | Absorption max. | Emission max. |
|---|---|---|---|
| DAPI | 350.25 | 358 | 461 |
| PI | 668.4 | 535 | 617 |
| $DiOC_6$ | 572.73 | 484 | 501 |
| Rhod 123 | 380.83 | 507 | 529 |
| JC-1 | 652.23 | 514 | 529 |
| Annexin V conjugates | Depends on fluorescent conjugate | | |
| AO | 301.82 | 500 | 526 |
| 7-AAD | 1270 | 546 | 647 |
| MitoTracker Red® CMXRos | 531.52 | 578 | 599 |
| Rhod-2, AM | 1123.96 | 550 | 571 |
| Hoechst 33342, 33258 | 623.96 | 352 | 461 |

excluded, as are cell fragments and endothelial cells; further stringency is added by ensuring that the person performing the cell counts is unaware of the treatments.

## Concluding remarks

The main objective of this article was to provide a brief overview of the methodologies available to study the cellular and molecular basis of stress-induced neurodegeneration. While our coverage is by no means exhaustive, we aimed to review each of the major approaches in current use, both in brain tissue and cell culture, and to discuss each of the methods in terms of their advantages and inherent drawbacks; it should become obvious to the reader that no single method can be considered to be definitive by itself, and investigators are encouraged to confirm results obtained one method with that from an alternative technique whenever feasible, in order to avoid from misinterpretation of results. We also attempted to discuss certain important aspects of experimental design in the hope that the use of standardized procedures will contribute to our increased understanding of stress-induced neuronal damage.

## List of abbreviations

| | |
|---|---|
| 5-HT | serotonin |
| 7-ADD | 7-aminoactinomycin D |
| AIF | apoptosis-inducing factor |
| AO | acridine orange |
| APAF-1 | apoptotic protease activating factor 1 |
| BDNF | brain-derived nerve factor |
| CA | field of hippocampus |
| DAPI | 4′-6-diamidino-2-phenylindole |
| $DiDOC_6$ | 3,3′-dihexiloxa-dicarbocyanine |
| EB | ethidium bromide |
| FACS | fluorescence-activated cell sorter |
| FGF-2 | fibroblast growth factor |
| FITC | fluorescein isothiocyanate |
| GFAP | glial fibrillary acidic protein |
| GR | glucocorticoid receptor |
| ISEL | in situ end-labeling |
| ISNT | in situ nick-translation |
| JC-1 | 5,5′,6,6′-tetra-chloro-1,1′,3,3′-tetraethyl benzimidazolyl-carbocyanine iodide |
| JNK | c-Jun N-terminal kinase |
| MK-801 | dizocilpine maleate |
| MPT | mitochondrial permeability transition |
| MR | mineralocorticoid receptor |
| $N_A$ | number per unit area |
| NeuN | neuronal-specific nuclear protein |
| NGF | nerve growth factor |
| NMDA | *N*-methyl-*D*-aspartic acid |
| NO | nitric oxide |
| $N_V$ | number per unit volume |
| PARP | poly(ADP-ribose) polymerase |
| PI | propidium iodide |
| PS | phosphatidylserine |
| PTP | permeability transition pore |
| Rhod | rhodamine |
| ROS | reactive oxygen species |
| TdT | terminal deoxynucleotidyl transferase |
| TGF | transforming growth factor |
| TuJ1 | neuron-specific class III beta-tubulin |
| TUNEL | Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling |
| URS | uniform random systematic |
| $\Delta\Psi_m$ | mitochondrial transmembrane potential |

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