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Cell death and proliferation in acute slices and organotypic cultures of mammalian CNS

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Key words: Brain slices, organotypic cultures, cerebral cortex, hippocampus, cerebellum, retina, cell proliferation, cell death, apoptosis

Abstract

Analysis of the interplay between cell proliferation and death has been greatly advantaged by the development of CNS slice preparations. In slices, interactions between neurons and neurons and the glial cells are fundamentally preserved in a fashion close to the in vivo situation. In parallel, these preparations offer the possibility of an easy experimental manipulation. Two main types of slices are currently in use: the acute slices, which are short living preparations where the major functions of the intact brain (including neurogenesis) are maintained, and the organotypic cultures, where the maturation and plasticity of neuronal circuitries in relation to naturally occurring neuronal death and/or experimental insults can be followed over several weeks in vitro. We will discuss here the main advantages/disadvantages linked to the use of CNS slices for histological analysis of neuronal proliferation and death, as well as the main findings obtained in the most popular types of preparations, i.e. the cortical, hippocampal, cerebellar and retinal slices

1. General concepts and purpose of this review

The balance between cell proliferation and death is funda- mental in several morphogenetic processes and ultimately determines the mass, shape and function of the various tissues and organs that form the animal body. Programmed cell death (PCD) is a gene-regulated process that plays important roles in several normal and pathological conditions (Kerr et al., 1972; Wyllie, 1980). One form of PCD is apoptosis, a form of ‘‘cell suicide’’ most often found during embryonic development, but also in normal cell and tissue turnover (Carrio et al., 1996; Oppenheim et al., 2001; Oppenheim, 1985; Polakowska et al., 1994; Zakeri and Lockshin, 1994, 2002). Although the nervous tissue is traditionally regarded as being fundamentally constituted by post-mitotic non-proliferating cells, analysis of cell proliferation and apoptosis has gained increasing importance mainly considering that: i. Proliferative and/or apoptotic events have been extensively character- ized not only during embryonic

development but also in several areas of the post-natal and adult brain (Alvarez-Buylla, 1997; Luskin, 1997; Gage, 1998; Lossi et al., 1998; Lossi and Merighi, 2003; Luskin, 1993), ii. Trophic factor deprivation often results in apoptotic cell death of target neurons (Bain et al., 1995; Bredesen, 1995; Raff et al., 1994; Yao and Cooper, 1995), and, iii. Links have been demonstrated between apoptosis, neuronal activity and signal transduction (Cunningham, 1982; Lee et al., 1993). Extensive work in the field has been carried out in vitro on isolated neurons and/or neural cell lines, whereas relatively few studies have been performed in vivo (Lossi and Merighi, 2003). These studies are highly demanding in technical and economic terms, and thus useful alternatives are usually sought, among which slice brain preparations have obtained increasing success. Slices from different brain regions are very important tools for studying the structural, physiological and pharmacological properties of neuronal circuits. They are relatively easy to prepare, and because they retain the cytoarchitecture of the tissue of origin, slices have clearly evolved as the predominant in vitro preparation used by electrophysiologists, and, although to a lesser extent, histologists, pharmacologists and biochemists. As slice-based assay systems provide good experi- mental access and allow precise control of extracellular environ- ments, they facilitate research aiming to establish clear correlations between structure and function, as well as plasticity of neuronal interactions under different experimental conditions. In addition, it is possible to adopt these ex vivo models for the screening of therapeutic molecules or novel genes. Two main types of slice preparations are currently in use in neuroscience research. The so-called ‘‘acute’’ slices (often simply referred to as slices) are short-lived preparations usually amenable for experiments in the range of a few hours. To overcome this obvious limitation to wider uses, attempts have been undertaken to establish alternative in vitro preparations that could be used for long-term studies. On these grounds, organotypic slice cultures were developed by taking advantage of previous work done on explant cultures of various anatomical origins. Since then, organotypic cultures have become an attractive alternative and complement to acute slices, and both the spectrum of possible culturing methods as well as the type of applications have been considerably expanded. Acute slices have come into use mainly as alternatives to electrophysiology studies in vivo, since the greater mechanical stability that these preparations provided over in vivo models and the control allowed over the composition of the extracellular environment have been particularly advantageous in permitting to perform successful patch clamp studies. In combination, these two characteristics have enabled detailed investigations on neuronal membrane properties, cellular actions of neurotransmitters, and synaptic mechanisms. In addition, the development of membrane permeant fluorescent indicators has made it possible to employ acute slices for studies on calcium homeostasis that is linked, among others, to cell survival (Bickler and Fahlman, 2004; Erin et al., 2003; Lee et al., 2003; Turner et al., 2007). Therefore this latter field of application has brought about a significant advancement of our understanding of cell death mechanisms in an experimental context where cell-to-

cell interactions are fundamentally preserved.

Organotypic cultures of brain slices have become an even more attractive tool for neurobiological studies of cell proliferation and death in vitro since their introduction over two decades ago. Originally developed by Gahwiler in 1981, cultured brain slices retain many essential architectural features of the host tissue, such as neuronal connectivity, cellular stoichiometry and glial–neuro- nal interactions (Gahwiler et al., 1997). The introduction of a relatively simple method of culturing hippocampal slices on semiporous membranes by Stoppini et al. (1991) has further increased their use in the study of the central nervous system (CNS) under normal and pathological conditions. With this approach, explanted tissues are layered onto a semiporous membrane and fed by medium on the underside of the membrane so that they are kept alive at the air–liquid interface inside a thin film of fluid. Prior to this development, it was necessary to grow cultures on coverslips in a chicken plasma clot inside test tubes placed in a slowly rotating drum that periodically immersed the cultures in medium, or to employ Maximov chambers under static conditions (Lindner and Grosse, 1982). Both methods are no longer commonly used since they are technically cumbersome and do not permit optimum access to the slice for manipulation.

Despite the above, comparatively few studies have employed acute slices and organotypic cultures as an alternative to the studies of the relationship between cell proliferation and death carried out in vitro on isolated neurons and/or in vivo. The purpose of this review is to provide comprehensive information about the possibilities to use these preparations as a tool to investigate neuronal proliferation and death from a histological and functional viewpoint, and to discuss the advan- tages/disadvantages of these approaches as compared to more traditional methods in vitro or in vivo.

1.1. Slice preparations for the study of cell death and proliferation in CNS

Brain slice models offer unique advantages over other in vitro approaches in that they largely preserve the tissue architecture and maintain neuronal activities with intact functional local synaptic circuitry for variable periods of time according to the different procedure of choice. Since it is possible to reduce the number of animals employed in different experiments, there is no need for lengthy animal surgery, or laborious monitoring of multiple physiological parameters following in vivo manipulation, organotypic cultures have gained increasing importance in basic as well as applied research during the past years.

1.1.1. Acute slices

1.1.1.1. General principles.

Acute slices have been prepared for a variety of CNS regions, and are usually obtained from young post- natal animals, but older animals have also been utilized. The preparation of acute slices is now a routinely carried out operation in numerous laboratories, and certain areas of the brain, i.e. the hippocampus, are particularly amenable to slicing. Briefly, the brain is dissected free of the skull and

rapidly placed into a saline solution whose composition resembles that of the cerebro-spinal fluid and is thus often referred to as artificial cerebro-spinal fluid (ACSF). Only with difficult samples (i.e. spinal cord) may it be necessary to employ special molds to hold tissue during the slicing procedure or agarose embedding. Slicing is performed with a vibrating microtome at high amplitude and very slow speed. Slice thickness varies according to specimen and the types of experiments, from 150 to 400 μm. It is commonly assumed that neuronal circuitry retains its stability in acute slices. However, it was recently demonstrated that mature hippocampal dendritic spines display a certain grade of plasticity (synaptogenesis) in relation to chilling and re- warming of ACSF (Kirov et al., 2004). In addition, synaptogenesis induced by exposure to ice-cold ACSF raises the concern that morphological correlates of LTP might be obscured, especially in mature slices (Bourne et al., 2007).

1.1.1.2. **Main disadvantages.**

Acute slices of conventional thickness present several problems when attempting to study ion channels and neurotransmitters in relative isolation. Several barriers including uptake mechanisms and degradative enzymes can slow or prevent the diffusion of drugs and hence hamper accurate pharmacological analysis. Pre- and post-synaptic components can be difficult to isolate in electrophysiological and imaging experi- ments.

1.1.2. **Organotypic cultures**

A number of pharmacological and genetic manipulations have been demonstrated to be successfully reproducible in organotypic slices (see for example Cho et al., 2004; Pringle et al., 1996; Ray et al., 2000). Therefore, not only has this approach been employed for study of normal brain development and function, but the availability of slice models that simulate essential features of in vivo neurodegen- eration has allowed for an array of treatments to be efficiently evaluated in normal or injured tissues without the complications derived from blood–brain barrier or metabolic stability. On the other hand, rearrangements of neuronal circuitry often occur in these slices, in parallel with substantial amount of cell death during the first days after explant, this being particularly important in the study of apoptosis. We will thoroughly discuss all these issues below.

1.1.2.1. **General principles.**

Organotypic cultures have been suc- cessfully established from a variety of CNS regions (see Table 1). Cultures are usually obtained from early post-natal, or, less frequently, from embryonic material. Early post-natal periods (P0 to P7) are ideally suited for culturing because the cytoarchitectonic fundamentals are already established in most areas, the brain is larger and easier to manipulate, and nerve cells are more likely to survive explantation. However, for the main purposes of our discussion, it should be kept in mind that the first 2 post-natal weeks in rodents are a critical period in relation to the peak of naturally occurring neuronal death (NOND) in several brain areas. This not only affects the degree of

cell death that one expects to detect in preparations, but is also one of the main reasons why early post-natal cultures from different CNS regions display very different behaviors regarding the differentiation of neuronal and glial cells (see below). Fetal tissues survive very well in slice cultures, but the organotypic organization is often distorted and/or poorly preserved, given that, earlier in development, massive neuronal migration and major rearrangements of neuronal circuitry still occur. Taking this into consideration, successful attempts have also been made to culture adult specimen (Hocke et al., 2007).

Tissue slice co-cultures have also been developed, which allow for the analysis of functional links across brain regions (see Table 1). As mentioned, the two earlier methods described by Gahwiler (1981) or Lindner and Grosse (1982) based on the use of either roller tubes or Maximov-type chambers have been shown to be difficult to get into routine use, bearing a large experimental variability due to thinning of tissues to a non-physiological cell monolayer. Therefore, most studies now utilize various modifications of the original membrane interface methods (Stoppini et al., 1991). The principle of membrane interface culture methods is to maintain brain slices on a porous membrane filter at the interface between medium and a humidified atmosphere. The medium provides adequate nutrition to tissues through the membrane via capillary action (Bergold and Casaccia-Bonnefil, 1997; Noraberg et al., 2005). It is important to note, however, that even in cultures prepared with the membrane interface method there are some properties that substantially differ from the characteristics acquired during the physiological brain maturation in vivo. For example, fluor- escent dye tracing of single neurons in organotypic hippocampal slices showed a more complex pattern of dendritic branching, and, consistently with this, a significant increase in the frequency of glutamatergic miniature synaptic currents, probably reflecting an increased number of total synapses (De Simoni et al., 2003). Similar results were obtained in cerebellum where the development of Purkinje neurons (PNs) was examined in organotypic cultures. In these preparations not only was it found that PNs in explants from P2 animals developed cytotypically in contrast to the organotypic appearance of P6 explants, but also that the PN dendritic tree was normal in the absence of excitatory neurotransmission and BDNF signaling (Adcock et al., 2004; Fenili and De Boni, 2003).

Table 1
Principal CNS areas employed for slice preparations. CNS regions

	References
Cerebral cortex	Annis et al., 1990, 1993; Baker et al., 1992; Behan et al., 1991; Brussaard and Baker, 1995; Caeser et al., 1989; Chattopadhyaya et al., 2004; Cheema et al., 2000; Corthesy-Theulaz et al., 1991; Dayer et al., 2008; Del Rio et al., 1996; Gotz and Bolz, 1989; Humpel and Weis, 2002; Ide et al., 1996; Jacobsen and Miller, 2003; Kierstein et al., 1996; Leiman and Seil, 1986; Molnar and Blakemore, 1999; Neely et al., 2007; Patz et al., 2003; Plenz and Kitai, 1996b; Prewitt and Wilson, 2007; Robertson et al., 1997; Romijn et al., 1988; Ross and Porter, 2002; Soares and Sotelo, 2004; Toran-Allerand, 1991; Vogt Weisenhorn et al., 1998; Wang and Johnson, 2007; Wirth et al., 1998; Yamamoto et al., 1989; Zhou et al., 1993; Zink et al., 2004
Striatum	Aleksandrova et al., 2001; Gutierrez et al., 2004; Hösli and Hösli, 1986; Humpel et al., 1996; Liu et al., 1995; Lyng et al.,

	2007; Mani et al., 2005; Neely et al., 2007; Ostergaard, 1993; Patz et al., 2004; Studer et al., 1994; Snyder-Keller et al., 2008; Wirth and Wahle, 2003; Zheng et al., 2005; Zhou et al., 1993
Hippocampus	Aleksandrova et al., 2001; Bruce et al., 1995, 1996; Bruckner and Grosche, 2001; Chen et al., 2004a,b; Chubakov et al., 1984; Coltman et al., 1995; De Simoni et al., 2003; Del Rio et al., 1991, 1996; Diekmann et al., 1994; Gahwiler, 1981; Gogolla et al., 2006a,b; Grimpe et al., 2002; Hafidi et al., 1999; Hoareau et al., 2006; Hocke et al., 2007; Holopainen and Lauren, 2003; Huuskonen et al., 2005; Ide et al., 1996; Ikegaya et al., 2002; Jensen et al., 2004; Kamada et al., 2004; Keller et al., 1983; Koyama et al., 2004; Laskowski et al., 2005; LaVail and Wolf, 1973; Ludwig et al., 2003; Marty and Onteniente, 1997; Mohajerani and Cherubini, 2005; Molloy and Kennedy, 1991; Morales et al., 2007, 2005; Muller et al., 1993; Newell et al., 1995; Nikonenko et al., 2003; Pomper et al., 2001; Sadgrove et al., 2006, 2005; Scharonowa and Chaspekow, 1980; Schousboe et al., 1993; Semino et al., 2004; Shetty and Turner, 1999; Shin and Chetkovich, 2007; Spahr-Schopfer et al., 2000; Vasilyev and Barish, 2004; Victorov et al., 2001; Viktorov and Khaspekov, 1976; Vizard et al., 2008; Wang and Johnson, 2007; Wilkins et al., 2006; Wise-Faberowski et al., 2005
Basal forebrain	Haas et al., 1998; Thomas et al., 1998; Wray et al., 1989
Thalamus	Magowan and Price, 1996; Okada et al., 2000; Zhao et al., 2003
Hypothalamus	Baertschi et al., 1982; Belenky et al., 1996; House et al., 1998; Ikeda and Allen, 2003; Israel et al., 2008; Maurer and Wray, 1997; Maurer and Wray, 1999; O'Neill et al., 2008; Palacios-Pru et al., 1991; Rusnak et al., 2007; Shimizu et al., 2008; Tominaga et al., 1994; Tominaga-Yoshino et al., 2007; Wray et al., 1988, 1993
Mesencephalon (whole)	Af Bjerkén et al., 2008; Larsen et al., 2008; Okawara et al., 2007
Inferior colliculus	Corse et al., 1999; Gerkema et al., 1999; Hafidi et al., 1995, 2004; Israel et al., 2003; Jourdain et al., 1998;
Pierre et al., 2001 Substantia nigra	Holmes et al., 1995; Jaeger et al., 1989; Neely et al., 2007; Ostergaard et al., 1996; Studer,
2001; Testa et al., 2005 Brainstem nuclei	Eustache and Gueritaud, 1995; Jones et al., 1995; Sanes and Hafidi, 1996; Thomas et al.,
1998; Zhao et al., 2004	
Cerebellum	Audinat et al., 1990; Ayoub et al., 2005; Bouslama-Oueghlani et al., 2003; Bruckner and Grosche, 2001; Calvet et al., 1985; Calvet and Calvet, 1988; Chedotal et al., 1996; Davids et al., 2002; Gähwiler, 1984; Julien et al., 2008; Knopfel et al., 1990; Kondoh et al., 2004; Lossi et al., 2004b; Mancini and Atchison, 2007; Nagata et al., 2006; Nagata and Nakatsuji, 1994; Nunzi et al., 2001; Nunzi et al., 2003; Ono et al., 1994; Savill et al., 2005; Schnadelbach et al., 2001; Soares and Sotelo, 2004; Tanaka, 2007; Yuan et al., 1998
Spinal cord	Baker et al., 1989; Bonnici and Kapfhammer, 2008; Corse et al., 1999; Hösli and Hösli, 1986; Krassioukov et al., 2002; Li et al., 2008; Rakowicz et al., 2002; Rickman, 1999; Shichinohe et al., 2008; Tolosa et al., 2008
Retina	Chen et al., 1997; Gorba and Wahle, 1999; Johnson and Martin, 2008; Kaempf et al., 2008a,b; Reh and Radke, 1988; Rickman, 1999; Viktorov et al., 2004, 2006
Cochlea	Andreeva et al., 2006; Ficarella et al., 2007; Khan et al., 2007; Kesser et al., 2007; Qi et al., 2008; Sobkowicz et al., 2002; Zheng and Gao, 1996
Olfactory bulb	De Marchis et al., 2001; Josephson et al., 2004
Co-cultures	Baratta et al., 1996; Dammerman et al., 2000; Franke et al., 2003; Gahwiler and Brown, 1985; Guido et al., 1997; Heine et al., 2007; Ichinohe et al., 2000; Jaumotte and Zigmond, 2005; Knopfel et al., 1989, 1990; Li et al., 1996; Mariani et al., 1991; Mingorance et al., 2004; Molnar and Blakemore, 1999, 1991; Neely et al., 2007; Oishi et al., 2004; Ostergaard et al., 1996; Plenz and Aertsen, 1996a,b; Plenz and Kitai, 1998, 1996a; Rennie et al., 1994; Snyder-Keller et al., 2001; Snyder-Keller, 2004; Woodhams et al., 1993; Woodhams and Atkinson, 1996

These exemplificative observations strongly suggest that cutting of projection axons, which inevitably occurs during slice preparation (Fig. 1), leads to a compensatory response over the course of the culture period, and that damaged axons have a chance to recover and reroute their processes to form new neural connections. Plasticity of connections was also confirmed in co-cultures. For example, when cerebellar slices were co-cultured with slices of the inferior olive some PNs became reinnervated by the olivary axons (Mariani et al., 1991). Plasticity of organotypic cultures is therefore a major difference with acute slice systems, where there are few changes in neuroanatomy (if any), as they are used within a few hours of preparation.

1.1.2.2. **Main disadvantages.**

Organotypic slice cultures are not exempt from drawbacks that somehow hamper their use in certain experimental paradigms. The first disadvantage of slice cultures is that not all areas of CNS are easily amenable to culture. The organotypic method is ideal for regions with a layered structure that can be aligned parallel to

the plane of slicing and is ideally confined inside the thickness of the slice itself (Figs. 1 and 2). The production of slice cultures from regions with significantly out-of- plane projections still remains challenging, primarily because a massive neuronal loss can follow the interruption of afferent/ efferent projections, with significant rearrangements in synaptic circuitry. Some interesting alternatives have been proposed to overcome these problems (Rehen et al., 2006). As mentioned, cultures can currently be produced mainly from embryonic or juvenile donor animals (typically up to 12 days post- natal). Production of slice cultures from older donors is possible. However, these cultures do not appear to be useful for drug screening purposes due to their low viability, given that only around 5–10% remain viable for 3–4 weeks (Bruce et al., 1995, 1996). Moreover, juvenile tissues are more resistant to ischemic damage than adult ones, and this issue needs to be considered when planning to use organotypic cultures for the study of ischemia (Lindroos et al., 2005).

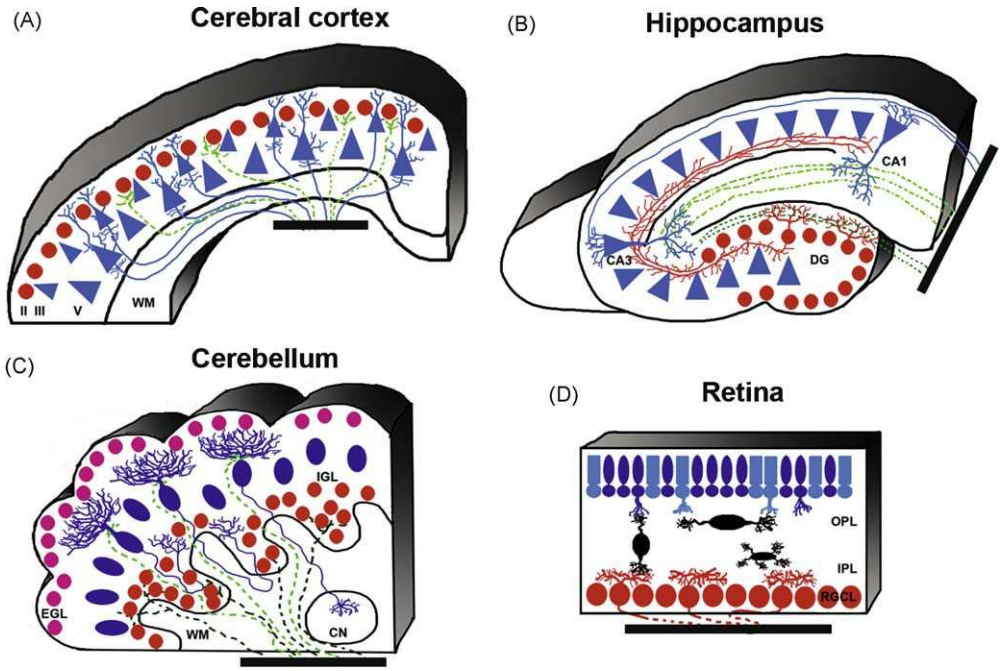

Fig. 1. Schematic representations of the main architectural features in cortical, hippocampal, cerebellar and retinal slices. A: In cerebral cortex slices the afferent fibre systems (green) primarily reach the granular cells (red circles) of layer II. Efferent fibres (light blue) mainly derive from large pyramidal neurons of layer V. Both are cut during slice preparation and thus degenerate in culture. B: In hippocampal slices the afferent fibre systems (green) mainly derive from entorhinal cortex and septum. Efferent fibres (light blue) are mainly derived from CA1 and CA3 pyramidal neurons. Both are cut during slice preparation and thus degenerate in culture. In organotypic preparations there is a significant sprouting of the mossy fibres (red), i.e. the axons of the granule cells (red circles). C: in cerebellar slices the afferent systems (i.e. the mossy – black – and climbing fibres—green) are degenerated. Efferent projections from PNs (blue) can be spared if nuclear neurons are comprised within the slice. Some PNs devoid of target become self- innervated. Cerebellar slices are generally produced at a stage in which the EGL is still present on the pial surface of the organ. The EGL contains

premigratory CGCs (fuchsia circles). Mature CGCs (red circles) form the IGL. D: in retinal slices the efferent fibres, i.e. the axons of the RGCs (red circles) are cut. Differentiation of the photoreceptor (light blue—rods; blue—cones) outer segments is impaired as a consequence of the lack of the pigment epithelium (not shown). Abbreviations: CN, cerebellar nucleus; DG, dentate gyrus; EGL, external granular layer; IGL, internal granular layer; IPL, inner plexiform layer; OPL, outer plexiform layer; RGCL, retinal ganglion cell layer; WM, white matter. Roman numerals indicate the layers of the cerebral cortex. The thick black segment outside the slice indicates the site of slice cutting from interconnecting structures. Projection fibres are represented by dashed lines, to indicate that they undergo progressive degeneration in culture.

The study of proliferation/apoptosis requires analysis of individual cells at relatively high magnification. In this respect, the tendency of the cultures prepared with the roller tube method to significantly and progressively thin in vitro is theoretically very advantageous. However, since the method is time consuming and repeated tracking of individual live cells is made very difficult, these types of organotypic preparations are, in practical terms, of limited value for the analysis of cell death. The air–medium interface method is preferable for repeated observations of live cells, since it is also possible to grow these cultures inside incubators adapted to the microscope stage. Nonetheless, some problems are still encountered in imaging individual cells at higher power (Fenili and De Boni, 2003). Another drawback of the roller tube method is the intense migration of glial cells that results in disruption of the architecture of the slice. Such a migration is strongly reduced in cultures obtained with the air–medium interface procedure. However, in this case, a carpet of astrocytes often grows on the top of the culture, unless DNA synthesis inhibitors are added (see Section 3.6). This astrocytic carpet makes the penetration of immunocy- tochemical reagents in labeling procedures more difficult. Lastly, organotypic slice cultures do not have a functional vascular compartment. Therefore, they cannot be used to accurately model the diffusion and mechanisms of action of systemically administered drugs, although this can be viewed as advantageous when trying to assess whether or not a drug can directly affect the nervous tissue independently from the presence of the blood–brain barrier. Regarding this latter issue, however, it must be taken into consideration that an in vitro model of the blood–brain barrier has recently been developed after treatment of organotypic cultures with basic fibroblast growth factor 2 (FGF-2 - Bendfeldt et al., 2007).

1.2. **Methods for the study of cell proliferation and death in CNS slice preparations**

The usefulness of CNS slices in a wide range of studies examining neuronal differentiation and fate is immediately apparent from the variety of methods that have been developed and/or adapted for assessing neuronal survival and death in these preparations. Whereas many of these methods have been derived directly from protocols that were originally developed in vivo, others have been specifically developed for in vitro use. In general terms, and irrespectively from

the above, the procedures available so far fall into two main categories: those that are suited to a direct observation of live preparations, and those that can only be employed once cultures have been fixed and further processed for histology. It is immediately obvious that the former only permit full exploitation of the potentials of slice preparations. This is because easy experimental manipulation of slices/cultures is accompanied by the possibility to follow dynamic responses over time, and to monitor changes by appropriate means.

1.2.1. Methods for the study of cell proliferation in live slices

The most popular means to label proliferating cells in live slice preparations is the addition of nucleotide analogues to the ACSF (for acute slices) or to the culture medium (for organotypic cultures) followed by immunocytochemical visualization (see

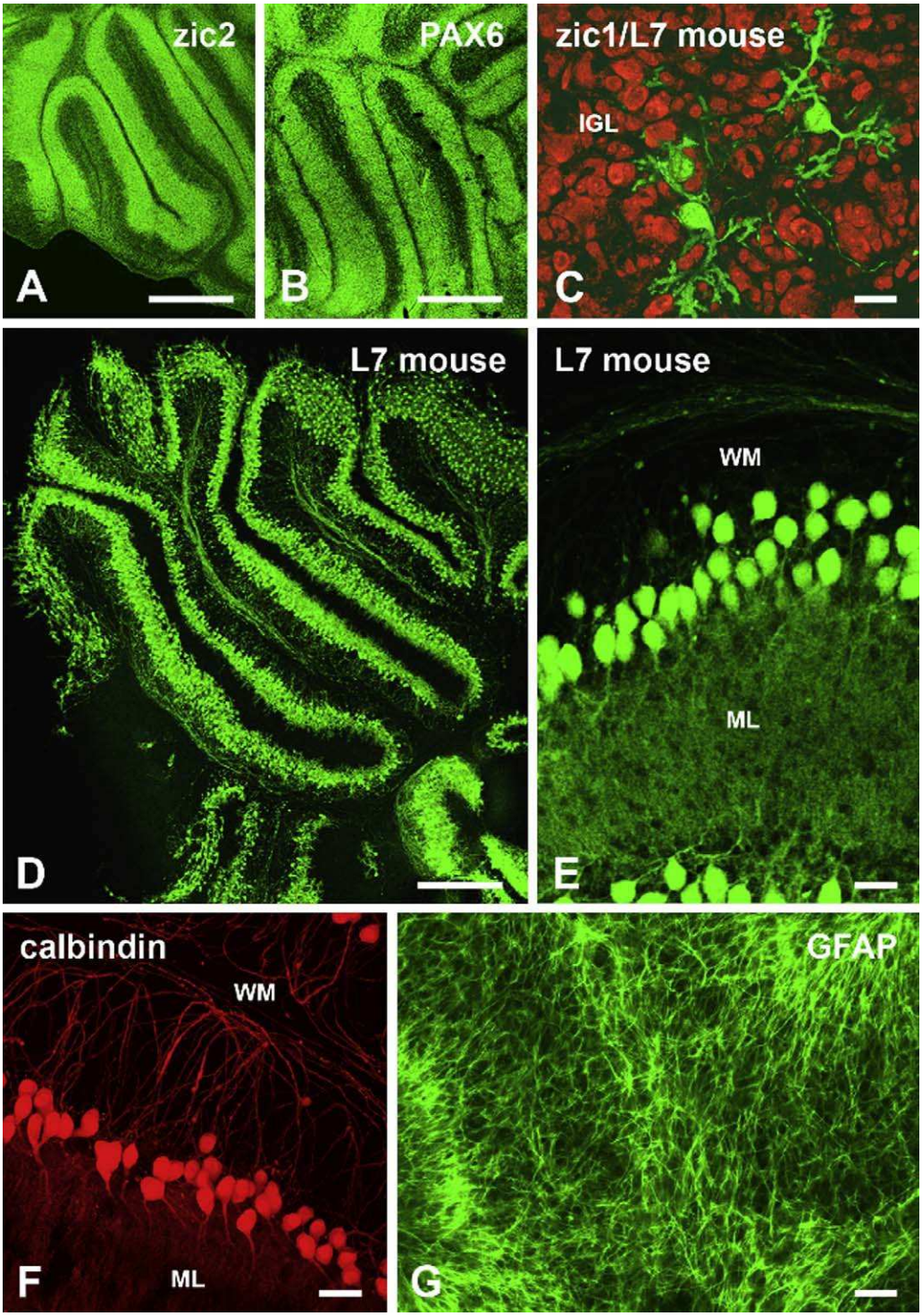

Fig. 2. Neurochemical characterization of post-natal murine cerebellar slices cultivated in vitro under static conditions. A–C: immunocytochemical labeling of CGCs. In C, nuclear zic1-immunoreactivity (red) is specifically expressed by CGCs within the IGL. Note the presence of two unlabeled ectopically placed PNs expressing GFP after genetic engineering (L7 mouse—green). D and E: Expression of GFP in the PNs of the L7 mouse. Note that the cultures are still immature since PNs are not yet aligned into a single row. F: labeling of PNs with an anti-calbindin antibody. The PN axons (top) are directed toward the white matter. G: GFAP immunolabeling reveals the presence of a carpet of astrocytes on the top of cultures. Abbreviations: GFP, green fluorescent protein; IGL, internal granular layer; ML, molecular layer; WM, white matter. Bars: A, B and D = 500 μm; C = 40 μm; E–G = 50 μm.

Becq et al., 2005; Liu et al., 2005; Nanobashvili et al., 2005; Raineteau et al.,

2006; Son et al., 2003; Zhang et al., 2007). These approaches have been very useful for analysis of cell proliferation, but only in fixed slices. Therefore, alternative protocols would be of immediate advantage to label and track proliferating cells when they are still alive.

To this purpose, dividing neural progenitor cells in subven- tricular zone (SVZ) and hippocampal slices were labeled at different time points by means of a set of retroviruses expressing fluorescent proteins. Authors were thus able to perform con- nectivity and lineage studies on newly generated cells directly in vitro (Laplagne et al., 2006; Noctor et al., 2002). One issue of care is that viral transduction of neural stem/progenitor cells can greatly affect differentiation (Falk et al., 2002). For instance, adenoviral transduction of neural progenitors induced a nearly complete astrocytic differentiation (97% of cells) and blocked neurogenesis (Hughes et al., 2002). Furthermore, several drawbacks are associated with viral vectors, such as cytotoxicity, limited transgene capacity, and difficulties in producing pure and high-titre stocks. As an alternative, non-viral transfection with FuGene6 was used to genetically manipulate neural stem/progenitor cells, without adversely affecting their growth or perturbing lineage selection (Tinsley et al., 2006). Such a method is valuable for examining the molecular mechanisms of cell fate determination in vitro and is potentially suitable for slice studies. Another protocol of potential interest is based on nucleofection, an improved method of cell electroporation (Gartner et al., 2006), given that conventional electroporation has already been successfully employed to label single neurons in hippocampal slices (Haas et al., 2001).

1.2.2. **Methods for the study of cell proliferation in fixed slices**

Once slices are fixed they can be directly processed as whole mount preparations or further cut to obtain cryostat or paraffin sections. Whereas the first approach allows for a direct examina- tion of the entire slice, and thus an easier correlation with previous electrophysiological and/or pharmacological manipulations, it is often difficult to get a proper penetration of immunocytochemical reagents into the entire depth of tissue. We have successfully overcome this problem by using very long incubation steps in immunocytochemical procedures and by adding the membrane permeabilizing detergent Triton X100 to incubation buffers. Alternatively, one can cut microtome sections of the original slice, although with this approach it is generally more difficult to correlate the histological data with previously performed experi- ments. We have been able to obtain good immunocytochemical labeling of paraffin embedded acute slices, but not of organotypic cultures. The latter, in fact, become distorted during the embed- ding procedure and are thus difficult to be sectioned along specific spatial planes. Irrespectively of the procedure employed, direct microscopic observation of mitotic cells is clearly insufficient for a correct

estimation of the proliferating index (percentage of dividing cells) due to the (relatively) short duration of the M phase of the cell cycle and asynchronies of mitoses. Therefore, the methods routinely in use rely on the possibility of: i. Labeling proliferating cells during the S phase of their cycle by incorporation of exogenously administered nucleotide analogues into the newly synthesized DNA when slices are kept alive (see above); ii. Directly visualizing in situ certain molecules that are expressed during different phases of the cell cycle once the cell is committed to division, and are therefore regarded as specific markers of cell proliferation (Table 2). Among the former, methods based on the use of nucleotide analogues such as 5-bromodeoxyuridine (BrdU) or 5-iododeox- yuridine (IdU) that are specifically incorporated into the DNA and subsequently visualized by immunocytochemistry are highly specific and allow for a precise identification of the proliferating cells and a correct estimation of the proliferating index. Non- radioactive BrdU labeling is now generally employed as the technique of choice because of its simplicity and lack of safety restrictions linked to the handling of radioactive material (Miller and Novakowski, 1988; Del Rio and Soriano, 1989; Soriano et al., 1991, 1993). Several anti-BrdU monoclonal antibodies are now commercially available that can be successfully employed to label proliferating cells in situ. In addition, some other antibodies that allow for the specific detection of BrdU or IdU have been successfully employed in vivo for time window experiments (Belecky-Adams et al., 1996; Bravo and Macdonald-Bravo, 1987; Kondo and Makita, 1996; Lossi et al., 2002b; Miller et al., 1991; Miller and Novakowski, 1988; Sato et al., 1995; Yanik et al., 1992). Application of BrdU labeling has been mainly directed to organotypic cultures, but very likely, similar experiments can be carried out on acute slices, although there are few examples of this type of application. Similarly, time window labeling with two different DNA probes can also be used in slices. An interesting feature of BrdU/IdU labeling is that once the marker has been incorporated into the parent cell DNA, it is then equally divided between the two daughter cells. Therefore, the label can be traced over several cell generations, according to the cell cycle kinetic parameters and the duration of the experiment (usually referred to as days in vitro = DIV). By calculating the time necessary for the halving of the tracers into the newly generated

Table 2
Markers of cell proliferation[a].

Positive/negative markers of cell proliferation	Main characteristics and biological activity during cell cycle
PCNA/Cyclin (mAb PC10)	36 kDa polypeptide which acts as an auxiliary protein of DNA polymerase d and is expressed at the transition of the phases G_1/S of the cell cycle
Ki-67 nuclear antigen (MAbs Ki-67, KiS5, and mib-1)	Nonhistone protein containing 10 ProGluSerThr (PEST) motifs which are associated with high turnover proteins and which plays a pivotal role in maintaining cell proliferation. It is expressed in the G_1, S, G_2 and M phases of the cell cycle, but not in G_0
Phosphorylated histone H3	Histone protein specifically expressed during the G_2 and M phases of the cell cycle
Phosphorylated histone H2AX	Histone protein expressed in DNA repair of double strand breaks, and in the G_2 and M phases of the
cell cycle DNA polymerase a	Key enzyme in DNA synthesis whose levels of activity gradually decrease during development
Topoisomerase IIa (MAb KiS4) phases	DNA-modifying enzyme. The a isozyme has a role in cell proliferation, and is expressed in the S, G_2 and M
Repp-86 (MAb KiS2)	Proliferation-specific protein (p86) expressed exclusively in the S, G2, and M phases of the cell cycle.
Telomerase	Cellular reverse transcriptase that helps to provide genomic stability by maintaining the

	integrity of the chromosome ends, the telomeres
Casein-kinase 2[a]	Growth-related serine/threonine protein kinase
Transferrin receptor	Glycoprotein participating in transferrin uptake
Statin	Nuclear protein specifically expressed in quiescent (noncycling) G_0 phase cells
Argyrophilic nucleolar organizer region proteins (AgNORs)	Proteins that accumulate in highly proliferating cells. Expression is low for G_1 phase and high for S-G_2 phase

[a] Modified from Lossi et al. (2002a)—see reference for further details.

cells it is thus possible to obtain reliable information on the length of the different phases of the cell cycle (see for example Lossi et al., 2002b; Contestabile et al., 2008).

Immunocytochemical methods relying on the visualization of specific markers can be obviously employed as an alternative or in addition to BrdU/IdU immunolabeling after slice fixation. A number of molecules associated to different phases of the cell cycle have been characterized so far, and, in most instances, specific antibodies have been raised against them, allowing for in situ detection. Other molecules, which have also been proposed as cell proliferation markers, are more indirectly linked to cell cycle events and, in general terms, only offer a rough estimate of certain metabolic activities which may be increased during cell replication rather than on cell division itself (see Table 2).

1.2.3. Methods for the study of cell death in live slices

Studies of cell death in live cells are usually carried out with fluorescent probes. Most of these were originally developed for flow cytometry or use in vitro on isolated cell preparations. Under these conditions, there are no problems of dye penetration through cellular membranes, and background staining is totally absent or irrelevant. When applied to slice preparations, the thickness of the acute slice/organotypic culture becomes a limiting factor to the practical use of most of these stains, not only because it is possible that penetration is poor, but also because of background staining. For example, in organotypic cerebellar slices we have experienced some difficulties in proper labeling of dead cell with ethidium homodimer-2 (EthD-2) due to high background fluorescence. Therefore, the use many of these probes in slice imaging still remains subjected to verification and needs to be fully exploited.

1.2.3.1. Fluorescent nuclear stains.

Several fluorescent nuclear stains have been proposed as tools for the identification of dead cells in the fluorescence/confocal microscope (see Table 3). Basically, these dyes bind to DNA and can be divided into two main categories according to their capacity to penetrate (permeant dyes) or not (impermeant dyes) the intact plasma membrane of living cells. Since, if compared to healthy neurons, injured nerve cells have compromised membrane integrity, allowing otherwise membrane-impermeable molecules to pass through, impermeant dyes have been widely employed to the purpose of detecting dead cells in alive preparations.

Impermeant stains. The cell impermeant stain propidium iodide (PI) is by far the most commonly employed fluorescent dye in slice studies (Fig. 3A and B). Together with the less frequently used ethidium bromide (EtBr), ethidium homodimer-1 (EthD-1)

and EthD-2, these probes can be used to detect cells at late stages of the death process (D'Agostino et al., 2007; Mironova et al., 2007; Small et al., 1997). Ethidium dyes can be excited with mercury- or xenon-arc lamps or with the argon-ion laser, making them suitable for fluorescence and confocal laser-scanning microscopy. PI and EthD-2 are taken up by damaged cells and intercalated into DNA. Upon DNA binding they become intensely fluorescent and can be easily quantified using image analysis software. In our experience, it is also possible to employ the same culture preparation for repeated stainings at different intervals of time and subsequent quantitative analysis. The family of SYTOX dyes is made by cell-impermeant cyanine dyes that are particularly good dead-cell stains. SYTOX Green is virtually non-fluorescent when free in solution but becomes brightly fluorescent when binding to nucleic acids with excitation and emission peaks similar to fluorescein. SYTOX Blue can be excited by a mercury lamp, but, unlike many blue-fluorescent dyes, it is also efficiently excited by tungsten–halogen lamps and other sources that have relatively poor emission in the UV portion of the spectrum. SYTOX Orange has shorter-wavelength emission, as compared with PI, and it can be more efficiently excited by conventional rhodamine filter sets. In addition, SYTOX Orange has a far greater fluorescence enhancement upon binding DNA than PI, suggesting that it may have a higher sensitivity as a dead-cell stain. Although SYTOX stains have only been employed sporadically to stain dead neurons in slices (Umemiya et al., 1993; Umihira et al., 2002), they are of potential interest for further applications. We have successfully employed SYTOX Green to stain dead cells in cerebellar slices prior to fixation. Noteworthy fluorescence was retained in fixed material. The Zn^{2+} indicators Newport Green (NG), Fluozin-3 and Zinpyr-4 are non-toxic and impermeable to the membrane of healthy cells. These indicators have been successfully used to label apoptotic cells in a fashion similar to PI (Stork and Li, 2006).

Permeant stains. Hoechst 33342 (bisbenzimide) is an AT- selective, double strand DNA-selective cell permeant dye belong- ing to the Hoechst family, which is widely employed in cycle

Table 3
Fluorescent nuclear stains for dead cells.

Stain	Type of fluorescence	Membrane permeability	Suppliers	Notes
PI	Red	I	NA	We have compared labeling efficiency of PI (see also Fig. 3A and B) and EthD-2 or Dead Red™ in LIVE/DEAD Reduced Biohazard Cell Viability Kit #1 (Invitrogen) in organotypic cultures, and found considerably lower efficiency for EthD-2/Dead Red™, associated with stronger background staining
EthD-1	Red	I	NA	
EthD-2	Red	I	NA	
EtBr	Red	I	NA	
Newport Green	Green	I	Invitrogen, USA	All these Zn^{2+} indicators display both nuclear and cytosolic fluorescence
Fluozin-3	Green	I	Invitrogen, USA	
Zinpyr-4	Green	I	Neurobiotex, USA United States Biological, USA	
SYTOXBlue	Blue	I	Invitrogen, USA	Does not require UV excitation
SYTOX Green	Green	I	Invitrogen, USA	Higher sensitivity (?)
SYTOX Orange	Red/Orange	I	Invitrogen, USA	Can be excited by most common light sources, i.e. argon-ion laser
Hoechst 33342	Blue	P	NA	Fluorescence shift in dead cells
Acridine orange	Green/Orange-Red	P	NA	Nuclear stain for early apoptotic cells
YO-PRO-1	Green	P	Invitrogen, USA	
SYTO 13	Green	P	Invitrogen, USA	
SYTO 16	Green	P	Invitrogen, USA	
SYTO 17	Red	P	Invitrogen, USA	
DAPI		S	NA	

Abbreviations: I = impermeant; NA = not applicable, means that reagent can be easily obtained from different sources; P = permeant; S = semipermeant.

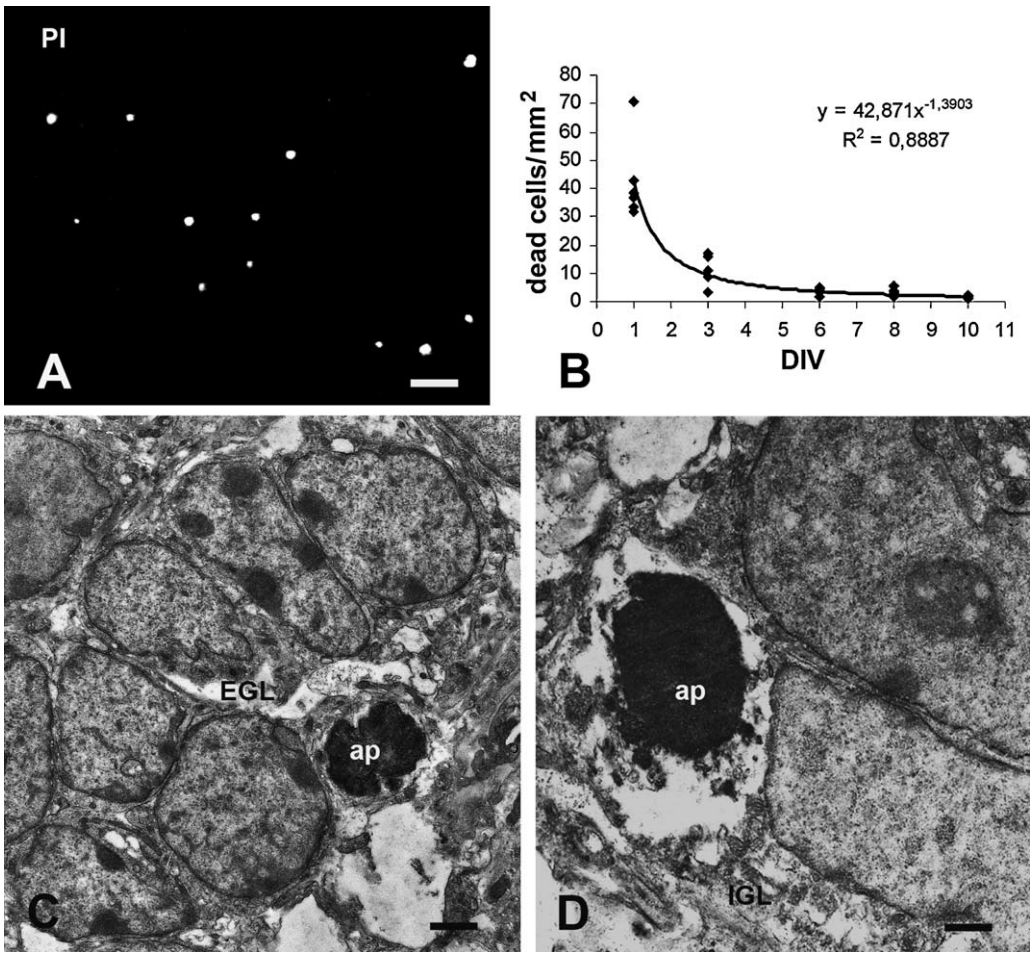

Fig. 3. Cell death in post-natal murine cerebellar slices cultivated in vitro under static conditions; A: PI staining of dead cells at 4 DIV. An area of 0.225 mm^2 is shown in the micrograph with 12 labeled nuclei. The extent of cell death varies considerably among different slices and PI stained nuclei are unevenly distributed in individual slices. Therefore accurate quantification and statistical analysis are mandatory to obtain consistent data from different sets of experiments. B = quantitative analysis of the course of cell death in slices after PI staining. Note that there is a peak of death immediately after explantation followed by a stabilization from 4 DIV onward. Each square represents a single experiment (one culture dish containing 3–4 slices). The number of dead cells has been calculated after automated counting of all PI stained nuclei in slices using the ImageJ NIH software. C and D: Apoptotic CGCs (ap) in organotypic slices display the same characteristic ultrastructural features observed in vivo, i.e. a highly electrondense nucleus with a pyknotic-like appearance as a consequence of chromatin condensation. Cytoplasm alterations become more evident as apoptosis proceeds to late stage (D). Abbreviations: DIV, days in vitro; EGL, external granular layer; IGL, internal granular layer; PI, propidium iodide. Bars: A = 50 μm; C and D = 1 μm.

studies. It shows an intense fluorescence when bound to the condensed chromatin in apoptotic cells. The rate of uptake in dying cells is correlated with low intracellular pH, and permeability of this probe theoretically allows for the staining of cells at very early stages of the apoptotic program, when plasma membranes are still intact (Dikkes et al., 2007; Hurtado et al., 2002; Kaul et al., 2003; Kress and Reynolds, 2005).

Acridine orange is another permeant probe which has been employed to distinguish live neurons from those undergoing apoptosis, by using its property to shift

fluorescence from green, at normal pH, toward brilliant orange-red, in the process of acidification. Further application of EtBr allows for detection of necrotic nuclei. An algorithm of automatic quantification based on image analysis was also developed for use with these two stains (Mironova et al., 2007). YO-PRO-1 iodide is a nucleic acid stain that crosses the plasma membranes of apoptotic cells brightly staining dying cells with a green fluorescence. YO-PRO-1 stain enters apoptotic cells at a stage when other dyes such as PI are still excluded (Fennell et al., 2006; Idziorek et al., 1995). SYTO dyes form a large family of cell permeant cyanine probes, with somewhat lower-affinity for nucleic acids, which passively diffuse through the membranes of most cells. These UV- or visible light-excitable dyes can be used to stain RNA and DNA in both live and dead cells. SYTO 13 was used in conjunction with PI to study glutamante-induced apoptosis in cerebellar granule cells (Ankar- crona et al., 1995).
4'-6-diamino-2-phenylindole (DAPI) associates with the minor groove of dsDNA. It shows a blue fluorescence that can be excited with a mercury lamp or the UV lines of the argon-ion laser. Binding of DAPI to dsDNA produces an intense enhancement of fluores- cence. Therefore, DAPI is often used as a generic nuclear counter- stain that allows easy identification of the highly condensed nuclei of apoptotic cells.

1.2.3.2. Fluorescent cytoplasm, mitochondrial and membrane stains. As for the nuclear stains, many of these dyes of potential interest for the study of cell death in tissue slices were originally developed for use in flow cytometric analysis. Basically, the stains of this group can be used to visualize apoptotic cells by taking advantage of certain metabolic alterations which (more or less specifically) take place during PCD. Cytoplasm stains. Reduction of the intracellular pH often occurs as an early event in apoptosis and may precede DNA fragmentation. The fluorescent carboxy dye SNARF-1 is a dual pH emission indicator which shifts from deep red (about 640 nm) in basic conditions to yellow-orange (about 580 nm) under acidic pH. Its cell permeant acetoxymethyl (AM) esther can be added directly to the incubation medium, and the dye becomes trapped inside the cells after hydrolyzation of the esther groups within the cytosol. Shifting of the emission spectrum of SNARF-1 allows for monitoring changes in the intracellular pH that occur in apoptosis (Meisenholder et al., 1996). Increased oxidative activity is another early event in PCD. Numerous dihydro, colorless, non-fluorescent leuco-dye deriva- tives of fluorescein and rhodamine are readily oxidized back to the fluorescent parent dye and can thus be used as fluorogenic probes for detecting apoptosis (Darzynkiewicz et al., 1994; France-Lanord et al., 1997; Hines and Allen-Hoffmann, 1996).
Mitochondrial stains. A number of fluorescent stains can be employed to monitor apoptosis by taking advantage of the disruption of mitochondrial homeostasis that occurs as a distinctive feature of the early phases of PCD. JC-1 (5,5',6,6'- tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocianine iodide) and JC-9 (3,3'-dimethyl-a-naphtoxacarbocyanine iodide) are two mitochondrial fluorescent dyes that shift from a green to a red fluorescent product as the mitochondrial membrane potential increases in relation to apoptosis. JC-1 has been employed to analyze mitochondrial changes in hippocampal neurons (Holts- berg et al., 1998). The fluorescent probes Mito Tracker Red CMXRos or Mito Tracker Green FM can be used to monitor apoptosis-induced mitochondrial membrane depolarization (Banki et al., 1996; Fernandez et al., 1995). Remarkably, Mito Tracker Red CMXRos survives aldehyde fixatives. Calcein AM is converted into calcein after entering the cell and displays a green fluorescence. Upon opening of the mitochondrial permeability transition

pore (MTP) the dye enters the mitochon- drial matrix. Given that MTP is involved in apoptosis, the uptake of calcein through the MTP can be used to study neuronal death (Gillessen et al., 2002). Other mitochondrial stains of interest are rhodamine 123 (Liu et al., 2001), and a number of carbocyanine dyes with short alkyl chains.

Plasma membrane stains. Annexin V is a 35.8 kDa protein that possesses a strong anticoagulant activity and belongs to a family of proteins with high calcium-dependent affinity for aminophospholipids, among which phosphatidylserine (PS - Dachary-Prigent et al., 1993). The latter is a major component of plasma membranes and is usually almost completely segregated to the cytoplasmic face of the membrane. Early on in apoptosis, PS is externalized as a means of recognition and uptake of apoptotic cells by phagocytes (Fadok et al., 1992). Translocation of membrane PS, which occurs in apoptotic cells (Dachary-Prigent et al., 1993), can be visualized in living cells by using the dye merocyanine 450 that undergoes a strong increase in fluorescence concurrently with the loss of cell membrane asymmetry that follows PS displacement. In addition, fluorochrome-Annexin V conjugates (Annexin V-fluorescein [FITC], Annexin V-Cy3, and Annexin V-Alexa Fluor 488) can be used for the visualization of the complexes between Annexin V and PS in apoptotic neurons (Zhang et al., 1998). We have experimented, albeit with limited success, the use of the Vybrant apoptosis Assay Kit #1 (Invitrogen, USA) to stain organotypic cerebellar slices. The kit employs a combination of SITOX Green and Annexin V-Alexa Fluor 488 to stain necrotic and apoptotic cells. Annexin V-Alexa Fluor 488 labeling could be observed under the fluorescence/confocal microscope for a very limited amount of time (less than half an hour) and did not survive fixation.

1.2.3.3. **Biochemical assays.**

Numerous means are available to assess cell death from a biochemical standpoint. Their discussion is beyond the purpose of this review that, as mentioned, mainly aims to analyze the histological applications of slice techniques for the study of cell death. We will only briefly mention below a few popular biochemical methods that have been used in support. Measurement of lactate dehydrogenase (LDH) activity in culture medium has been commonly used to quantify cell death in primary cultures (Koh and Choi, 1987) and organotypic slices (Bruce et al., 1995; Noraberg et al., 1999). The tetrazolium dye 3-(4,5-dimethlythiazol-2-yl)-2,5-diphe- nyltetrazolium bromide (MTT) is metabolically converted from a yellow soluble dye to insoluble purple precipitates within the mitochondria of live cells. Therefore, quantitative analysis of MTT conversion is also suitable for assessment of cell viability in organotypic slices (Connelly et al., 2000; Eve et al., 2007).

1.2.4. **Methods for the study of cell death in fixed slices**

1.2.4.1. **Light microscopy.**

Fluorescent tracers. Fluoro-Jade is an interesting specific and selective tool to identify degenerating neurons undergoing degeneration in fixed preparations (Schmued et al., 1997). A peculiarity of this type of stain is that Fluoro-Jade not only labels neuronal cell bodies, but also dendrite and axon processes. Fluoro- Jade has recently been used for detection of dead cells in hippocampal organotypic cultures after endoplasmic reticulum stress (Lacour et al., 2007).

Immunocytochemistry. The number of molecular markers of apoptosis that can be immunocytochemically detected in tissue samples has exponentially grown in the past years, and reviewing this matter is outside the scope of the present discussion. Table 4 simply gives an overview of the most widely employed reagents together with critical notes about specificity for apoptotic cells. The table does not take into consideration the Bcl-2 family members given the complexity and, at times, counteracting actions of these proteins in nerve cell survival (see Lossi and Merighi, 2003).

Molecular biology. DNA fragmentation in small size oligomers is a biochemical hallmark of apoptosis. Nucleosomal DNA ''ladders'' can be appreciated after electrophoretic separation of DNA in agarose gels and are a consequence of the action of DNA nucleases on the chromatin, to produce double-stranded DNA fragments with a size reflecting oligomers of the nucleosomes (Wyllie et al., 1984). An important advance in the detection of dying cells in situ by light and fluorescence microscopy was the development of a series of molecular biology protocols which can be generally referred to as in situ end-labeling (ISEL) techniques. These techniques were originally developed for studies in preparations other than slices, and, with the exception of the TUNEL procedure (see below) they have only rarely been used in thick tissue slices. Each of the ISEL techniques utilizes a different DNA modifying enzyme to attach labeled nucleotides to the free ends of fragmented DNA. In fact, cleavage of the DNA during the apoptotic process may yield double-stranded mono- and/or oligonucleosomes of low molecular weight, as well as ''nicks'' (single strand breaks) in high molecular weight DNA. These DNA strand breaks can be detected by enzymatic labeling of the free 3^0-OH termini with modified nucleotides, usually dUTP linked to a fluorochrome, biotin, or digoxigenin (DIG). The ISEL techniques include the in situ nick translation with DNA polymerase I (Gold et al., 1993; Jin et al., 1999) or unmodifyed T7 polymerase (Wood et al., 1993), the end labeling with terminal

Table 4
Main molecular markers of apoptosis in use for immunocytochemical stain.

Molecular marker	Example references	Suppliers	Notes
Cleaved-Caspase 1	Arai et al., 2006; Taylor et al., 1997; Zhang et al., 2003a	Cell Signaling Technology, USA Santa Cruz Biotechnology, USA	Involvement in neuronal apoptosis is still under debate
Cleaved-Caspase 3, and 9	De la Rosa and De Pablo, 2000; Friedlander and Yuan, 1998; Kuida, 2000; Lindholm and Arumae, 2004; Springer et al., 2001	Abcam, UK Cell Signaling Technology, USA Santa Cruz Biotechnology, USA	Caspase-3 (CPP-32, Apoptain, Yama, SCA-1) is a critical executioner of apoptosis, as it is either partially or totally responsible for the proteolytic cleavage of many key proteins such as the nuclear protein PARP-1. Activation of caspase-3 requires proteolytic processing of its inactive zymogen into activated p17 and p12 fragments Caspase-9 (ICE-LAP6, Mch6) is an important member of the caspase family. Upon apoptotic stimulation, cytochrome c released from mitochondria associates with the 47 kDa procaspase-9/Apaf 1. This complex processes procaspase-9 into a large active fragment (35 kDa or 17 kDa) and a small fragment (10 kDa). Cleaved caspase-9 further processes other caspase members, including caspase-3 and caspase-7, to initiate a caspase cascade, which leads to apoptosis Immunostaining with antibodies raised against the cleaved fragments of these two caspases detects apoptotic cells with very high specificity

Cleaved-Caspases 6, 7, 8, and 12	Allsopp et al., 2000; Guo et al., 2006; Hartmann et al., 2001; Horowitz et al., 2004; Ivins et al., 1999; Jin et al., 2001; LeBlanc et al., 1999; Nakagawa et al., 2000; Neekhra et al., 2007; Russo et al., 2004; Sharma and Rohrer, 2004; Siniscalco et al., 2008; Velier et al., 1999; Wootz et al., 2004; Zhang et al., 2000, 2003b, 2006; Zhao et al., 2008a	Abcam, UK BD Pharmingen, USA Cell Signaling Technology, USA	Data are mainly referred to neurodegeneration and/or injury
Cleaved PARP-1	Koh et al., 2005; Lossi et al., 2004a	Cell Signaling Technology, USA	PARP-1, a 116 kDa nuclear poly (ADP-ribose) polymerase, is involved in DNA repair in response to environmental stress. Cleavage of PARP-1 facilitates cellular disassembly. Cleaved PARP-1 immunostaining is a reliable marker of cells undergoing apoptosis
IAPs Smac/Diablo	Lotocki et al., 2003; Perrelet et al., 2004; Siegelin et al., 2005	Cell Signaling Technology, USA Novus Biologicals, USA R&D Systems, USA	Smac/Diablo is a 21 kDa mammalian mitochondrial protein that functions as a regulatory component during apoptosis Upon mitochondrial stress, Smac/Diablo is released from mitochondria and competes with caspases for binding of IAPs (inhibitor of apoptosis proteins)
FADD	Fuchs et al., 2005; Kim and Park, 2005; Wu et al., 2005	Cell Signaling Technology, USA Stressgen, Canada Santa Cruz Biotechnology, USA	FADD (Mort 1) functions as an important adaptor in coupling death signaling from membrane receptors to caspase 8
a-Fodrin	Emgard et al., 2003; Rohn et al., 2001; Springer et al., 1999	Cell Signaling Technology, USA	Data are mainly referred to neurodegeneration and/or injury
AIF	Plesnila et al., 2004; Sanders and Parker, 2002; Zhang et al., 2002	Cell Signaling Technology, USA	AIF is an ubiquitously expressed flavoprotein that plays a critical role in caspase-independent apoptosis. AIF is normally localized to the mitochondrial intermembrane space and released in response to apoptotic stimuli
c-Myc	Ferrer et al., 2001; Rong et al., 2003; Ueno et al., 2006	Santa Cruz Biotechnology, USA	The proto-oncogene c-myc encodes the transcription factor c-Myc, which is of great importance in controlling cell growth and vitality. Reduction of c-myc expression and its inappropriate expression can be associated with cellular apoptosis
Daxx	Junn et al., 2005; Raoul et al., 2005; Shinoda et al., 2003	Cell Signaling Technology, USA	Loss of Daxx in mice leads to embryonic lethality with extensive developmental apoptosis, suggesting a role for Daxx directly or indirectly in suppressing cell death

deoxynucleotidyl transferase (TdT) (Gavrieli et al., 1992; Gold et al., 1993), and the ligation of DIG-labeled double stranded DNA fragments with T4 DNA ligase (Didenko and Hornsby, 1996; Lossi et al., 1998).

1.2.4.2. **Ultrastructural analysis**. Apoptosis is characterized by a series of stereotypical ultrastructural changes in cell morphology and thus, at least in theory, electron microscopy (EM) represents the ideal tool for an unequivocal identification of apoptotic cells (Fig. 3B and C). Changes in cell ultrastructure during apoptosis affect both nuclear and cytoplasm morphology. Nuclear changes include chromatin condensation, blebbing of nuclear membranes, and fragmentation of the nucleus into highly electrondense apoptotic bodies. Cytoplasmic changes consist of condensation and fragmentation of the cell body into membrane-bound vesicles, which contain ribosomes, morphologically intact mitochondria and nuclear material. It should be stressed that all these changes take place at rather late stages of the apoptotic program and that apoptotic cells are rapidly cleared from tissue by phagocytes (Lossi and Merighi, 2003). This explains why ultrastructural analysis is often insufficient for the detection of apoptosis in tissue sections unless a very large population of cells is undergoing PCD. Therefore, although electron microscopy is likely the most specific tool for the identification of apoptotic cells in situ, it suffers several pitfalls mainly related to its dramatically low degree of sensitivity. An additional problem, when using slice preparations, is the preservation of proper ultrastructure for EM analysis. We have been able to properly process organotypic cerebellar slices to this purpose (Fig. 3C and D), but not acute slices.

2. Study of cell proliferation and death in acute slices

In acute slice preparations of most brain regions, neuronal functions are preserved for few hours only. Since the effects of growth factors or neurotoxic agents are often manifested beyond this time scale acute slices have only occasionally been employed for studies on cell proliferation and death, although attempts have been made to develop alternative protocols allowing the slices obtained from newborn rodents to stay alive for at least 24 h (Metzger et al., 2005). The literature specifically related to analysis of proliferation/ death in acute slices is discussed below.

2.1. Neurogenesis and NOND

Despite short viability of acute slices, BrdU labeling and electrophysiology experiments demonstrated that adult (P60-70) hippocampal neurogenesis (see also Section 3.2.1) is main- tained (Snyder et al., 2001), and recent observations have suggested a critical role of fibroblast growth factor receptor 1 (FGFR1) in the generation of neuronal progenitors in the dentate gyrus (DG) (Zhao et al., 2007). A direct analysis of NOND in acute slices has been performed only occasionally. Very initial studies on slice preparations of the rat medulla oblongata and pons maintained for 5 h in vitro demonstrated a significant occurrence of apoptotic cells, likely as a consequence of the cutting procedure (Hinrichsen, 1980). Apop- tosis has been observed also in acute spinal cord slices obtained after partial nerve injury (Moore et al., 2002). More recently, caspase-3 activity was studied in rat hippocampal slices, in parallel with the electrophysiological characteristics of extracellular responses to paired-pulse stimulation of Schaffer's collaterals in the CA1 subfield (Kudriashova et al., 2008). Maximal caspase-3 activity was observed in slices with low responsiveness to single afferent stimulation, a fact indicative of decreased efficacy of synaptic interactions. This phenomenon was unrelated to depres- sion of neuronal excitability, since paired-pulse stimulation increased the synaptic efficacy in response to a second stimulus, thus restoring population spike amplitudes to normal values. Moreover, in ''damaged'' slices with impaired spike generation, caspase-3 activity was close to the normal level seen in ''healthy'' slices. Authors have interpreted these data as suggestive that high caspase-3 activity in hippocampal slices is involved in main- tenance of synaptic plasticity but not necessarily related to apoptosis.

2.2. Experimental cell death

In the rat cerebellar slice preparation, exposure to hypoxia elicited by a 30 min exposure to ACSF continuously gassed with 95% N_2: 5% CO_2 induced a characteristic type of toxicity of PNs resembling an equally characteristic type of excitotoxic-mediated ''dark cell degeneration'' (Barenberg et al., 2001). Morphologically, PNs exhibited a marked rounded appearance with cytoplasmic darkening, nuclear condensation and cytoplasmic vacuoles. Using gel electrophoresis, genomic DNA obtained from slices appeared to be fragmented into

small size oligomers. However, PNs failed to exhibit apoptotic bodies, evidence of phagocytosis, spherical- or crescent-shaped chromatin aggregations, or TUNEL-positive stain- ing. Conversely, ultrastructural analyses of the cerebellar granule cells (CGCs), revealed the presence of apoptotic bodies and discrete spherical collections of chromatin clumping as well as phagocy- tosis, suggesting that the oligonucleosomal-sized DNA fragments were primarily derived from these neurons.

3. Study of cell proliferation and death in organotypic slices

The literature specifically related to analysis of neuronal proliferation/death in organotypic slices is discussed below, with particular emphasis on cerebral cortex, hippocampus, cerebellum, and retina. Other CNS areas and glia will also be mentioned briefly.

3.1. Cerebral cortex

3.1.1. Neurogenesis and NOND

Development of the cerebral cortex starts with the generation of post-mitotic neurons from proliferating neuroblasts in the ventricular zone (VZ). To this, a migratory phase follows that eventually leads to the shaping and differentiation of the mature layered cortex. Apoptosis also occurs during the entire process, and the balance of proliferation and death ultimately determines not only the fate of individual cells, but also and more importantly, the wiring of cortical circuitry (see Caviness et al., 1995; Caviness and Takahashi, 1995; Rakic, 1995; Rakic and Caviness, 1995). Our knowledge of cell death, cell cycle kinetics, and migration of post- mitotic cells from the VZ in organotypic slice cultures under normal conditions is quite extensive, since cultures obtained from E14 embryos maintain the morphology of the different zones of the neocortical wall (see Haydar et al., 1999a,b; Martínez-Cerdeño et al., 2006). In addition, by using DiI-labeled slices it was possible to study the assembly of the radial glia in parallel to the division of the parent cells (Miyata et al., 2001). Analysis of cell death with the TUNEL method has demon- strated that there is very little programmed cell death (about 5%) in the VZ up to 3 DIV, whereas it appears that the duration of the cell cycle almost doubles as compared to the in vivo situation. By calculating the clearance rate of apoptotic cells in cultures, it was concluded that cells die as a result of the injury during the preparation of the slice (Fig. 1A) and that, similarly to in vivo, there is a very low level of further cell death in the culturing phase (Haydar et al., 1999a; Takahashi et al., 1999). In keeping with these observations, it has been very recently shown that a network of synchronized spontaneous bursts is maintained in slices. These bursts activate ionotropic glutamate receptors and thereby promote neuronal survival (Heck et al., 2008).

Finally, it is of interest that human cortical tissue slices obtained by autopsy have been shown to survive well in vitro up to 78 DIV thereby permitting a series of experimental manipulations to be performed, including cell transfection (Verwer et al., 2002).

3.1.2. Experimental cell death

3.1.2.1. **Excitatory amino acid neurotoxicity**. Cortical slices have been extensively employed for the study of excitatory amino acid neurotoxicity. Effects of n-methyl-D-aspartate (NMDA), kainate, and a-amino-3-hydroxy-5-methylisoxazole-4-propionic acid hydrate (AMPA) on neuronal death have been characterized by the use of PI, Fluoro-Jade, and measurement of LDH activity, and results showed a good correlation between different markers, and with the observations in vivo (Hasegawa et al., 1998; Yoshikawa et al., 1998; Zimmer et al., 2000). In cultures it was also possible to study the modulation of excitotoxicity. After different types of pharmacological manipulations, it was demonstrated that several drugs are capable of inducing death by activation of NMDA/AMPA receptors and/or their subunits (Nakano-Okuda et al., 2006; Shirakawa et al., 2005, 2006; Wang et al., 2005). For example, the ability of tetraethylammonium to enhance ischemic neuronal death suggests that BK(Ca) channels constitute an endogenous system to protect cortical neurons from ischemic injury, leading to prevention of NMDA receptor over-activation (Katsuki et al., 2005). Additionally, it was shown that tumor necrosis factor (TNF) a and glutamate can act synergistically to induce neuronal cell death. On these bases, it was speculated that the TNF a potentiation of glutamate neurotoxicity (through the blockade of glutamate transporter activity) may represent an important mechanism of neurodegeneration associated with neuroinflammation. In general terms, the neurotoxicity through glutamate-NMDA receptors (or oxidative stress—see below) appears to be dependent upon CREB (Zou and Crews, 2005) and NF-kappaB DNA transcription (Zou and Crews, 2006). Excitatory amino acid neurotoxicity mainly induces apoptosis, although necrosis was also observed, according to different experimental paradigms. Focal swellings (varicosities) along dendrites are considered to be a hallmark of acute excitotoxic neuronal injury. NMDA-induced necrotic neuronal death in slice preparations was largely suppressed by a blocker of the volume- sensitive outwardly rectifying (VSOR) Cl-channel. VSOR Cl-channels exert dual, reciprocal actions on neuronal excitotoxicity by serving as major anionic pathways both for varicosity recovery after washout of an excitotoxic stimulant, and for persistent varicosity formation under prolonged excitotoxic insults leading to necrosis in cortical neurons (Inoue and Okada, 2007).

3.1.2.2. **Ischemia.** Organotypic cortical cultures have been widely employed to study the consequences of ischemia-induced injury during perinatal development by oxygen and glucose deprivation (OGD). It was demonstrated that OGD induces apoptosis and a profound dysfunction in subplate neurons and layer V pyramidal cortical neurons, and that NMDA receptors play a critical role in early ischemia-induced neuronal damage (Albrecht et al., 2005). Other studies shed some light on the molecular pathways that are activated during the apoptotic process. These studies showed that Bad phosphorylation plays a prominent role in the activation of the survival factor Akt with subsequent reduction of cytochrome c- mediated

apoptosis (Hirai et al., 2004). Exposure of cortical slices to 40%, instead of 20%, O_2 (the usual concentration that is reached in 5% CO_2 incubators) significantly improved the overall thickening of slices, cell production, layer formation. It also provided better spatial resolution by preventing the loss of transparency that accompanies cell death (Miyata et al., 2002). These effects have been explained by postulating a release from the VZ of protective factors against OGD (Cavaliere et al., 2006). It was also hypothesized that the SVZ neuroblasts respond to 40% O_2 with a more intense dynamic migratory and proliferative behavior (Zhang et al., 2007). Although excitotoxicity and OGD may share some of the cellular mechanisms eventually leading to death, there are clearly some differences in the pathways that are activated under these two different experimental conditions. For example, cytotoxicity of NMDA on rat cerebrocortical slice cultures was potentiated by addition of glycine or d-serine. In contrast, apoptosis induced by OGD was not affected by exogenous glycine or d-serine, although blockade of NMDA receptors by MK-801 abolished cell death (Katsuki et al., 2007).

3.2. Hippocampus

3.2.1. Main histological features

The hippocampus is by far the best characterized area of the brain in slice cultures since it has been subjected to extensive multidisciplinary investigations (see Table 1). Readers are thus referred to existing reviews specifically dealing with this issue (e.g. Gahwiler et al., 1997; Holopainen, 2005), since we will only briefly mention here some fundamental concepts which will help put things in the right perspective to understand the usefulness of this type of preparation in the histological analysis of neuronal cell death and proliferation. Typical hippocampal slice cultures are obtained from P3-9 rodents, but older rats (P30) have also been employed. Cultures can be maintained in vitro for several weeks, during which the developmental maturation continues ex vivo, in parallel with characteristic morphological rearrangement. During the prepara- tion afferent fibers are cut and, thereafter, degenerate (Fig. 1B). Although one might expect that synaptic rearrangements also occur under this condition, few such changes have been observed. All neuronal types (the granule cells, the pyramidal neurons and the interneurons) that exist in vivo are well preserved in cultures and, broadly speaking, maintain their morphology. Thus, mimick- ing the in vivo situation, the mossy fibers, i.e. the DG granule cell axons, are never seen to cross the CA3–CA1 border (Zimmer and Gahwiler, 1984). Similarly, CA1 pyramidal cells make few contacts with other CA1 or CA3 pyramidal cells (Gahwiler, 1984). Some differences have however been reported as regard to the dendritic arborization of the granule cells that is significantly reduced in vitro, likely as a consequence of the deafferentation. Another significant difference is the collateral sprouting of the mossy fibers which progressively increases in the molecular layers of the DG. The degree of sprouting depends on the position along the septohippocampal axis from which individual slices have been obtained, and is considerably reduced in hippocampal–entorhinal cortex co-cultures. Reorganization of the

processes of the granule cells is likely at the basis of the progressive increase of their excitatory activity as cultures become older. Similar changes have been observed after lesion and excitotoxic neuronal damage in vivo. Immediately after explantation, the density of synaptic contacts decreases, but after a few DIV the development of synapses essentially parallels synaptogenesis in situ. Pyramidal cells display normal synaptic transmission and plasticity. Branch- ing of neuronal processes and connections among neurons increase with time in culture, and come very close to the situation in vivo. In particular, confocal microscopic analysis demonstrated that slices cultured for 1, 2, or 3 weeks in vitro were developmentally equivalent to brain slices dissected at post-natal day 14, 17 or 21, respectively, in the number of primary branches, the total length of neurons, outgrowth of apical dendrites and spine density (De Simoni et al., 2003). The morphological characteristics of oligodendrocytes are very similar to those in vivo. Conversely, the layer-specific distribution of astrocytes is lost, and astrocytes appear not to reach full maturation. Microglial cells were shown to be activated following explantation, but they progressively return to a resting state after about a week in vitro (Holopainen, 2005).

3.2.2. **Neurogenesis and NOND**

In the DG cell proliferation and apoptosis occurs throughout lifetime. The relationship between the two processes is complex (see for example Kempermann, 2002; Lehmann et al., 2005). In vivo, it was originally assumed that the number of proliferating neurons correlates with that of those undergoing apoptosis not only during development, but also throughout life and in different experimental conditions (Gould et al., 1991; Heine et al., 2004a). However, it was subsequently shown that the two events are, in part, independent from each other (Heine et al., 2004b). Obviously, it would be advantageous to analyze the relationship of the two processes in slice cultures because one has, for example, the possibility to experimentally manipulate the system to specifically block proliferation or apoptosis. However, analysis of cell proliferation and death in hippocampal slices is complicated by the notion that, when prepared from P6-7 rats, DG granule cells are still proliferating whereas the CA1–CA3 pyramidal neurons and the interneurons are post-mitotic (Holopainen, 2005). Also, neurogen- esis within the DG appears to occur normally after BrdU/NeuN double labeling, and newly generated cells proliferated for at least 4 weeks following slice labeling (Raineteau et al., 2004). The occurrence of normal neurogenesis was confirmed in cultures obtained from transgenic animals where it was possible to track the migration pathways of neuronal precursors over a period of 45 days (Nieto et al., 2001; Sun et al., 2001).

More Cajal–Retzius cells (a population of transient pioneer neurons in layer I of the cortex that play essential roles in corticogenesis) were reported to survive than in vivo, where most of these cells undergo cell death during the third post-natal week (Del Rio et al., 1996). Apart from this study, little attention has been paid up to now to NOND in hippocampal slices, as the majorities of reports have been focused

on analysis of cell death induced by an array of experimental insults.

3.2.3. Experimental cell death

An interesting feature to take into consideration when critically considering the results of the numerous types of experiments aiming to clarify the mechanism(s) of experimental cell death in hippocampal organotypic cultures is the existence of a regional specificity in the response of neurons to the induction of cell death. Such specificity is indeed observed in most experimental para- digms, as will be apparent from the following discussion.

3.2.3.1. Serum deprivation and drugs affecting protein synthesis and cytoskeleton.

Serum deprivation is one of the most widely employed paradigms to induce death of several types of dissociated neurons in primary cultures. The inclusion of serum in culture medium is thus often found to be necessary for maintenance of neuronal cells, unless specifically formulated chemical defined media are employed. In hippocampal organotypic cultures, serum deprivation induced cell death within 6 h in DG granule cells and hilar interneurons, whereas neurons from other hippocampal regions were spared. Cell death was abolished by cycloheximide, a protein synthesis blocker, and depolarizing concentrations of kainate. These data indicate that protein synthesis-dependent PCD occurs in the DG upon trophic support withdrawal, and suggest that neuronal activity contributes to maintenance of certain hippocam- pal neurons in slices (Rivera et al., 1998). Given that a proper trophic support is provided, a variety of bioactive molecules and experimental manipulations have been employed to induce death in hippocampal slices. Susceptibility to cell death not only appears to be obviously dependant on the type of inducer employed, but it is also strictly related to the type of neurons and the stage of tissue maturation at the time of explant. For example, after 7 DIV cultures are subjected to apoptosis upon colchicine treatment, whereas at longer survival time (21 DIV) they become resistant (Kristensen et al., 2003). Similar results were obtained with a series of other cytoskeleton-disrupting drugs and lead to conclude that DG granules were primarily affected, but not the pyramidal neurons (Kim et al., 2002). Endoplasmic reticulum stress is another experimental condition leading to death of hippocampal neurons. Neuronal loss appears to affect the various areas of hippocampus (DG > CA1 > CA3) with different intensities, and to be associated with the activation of caspase 12 (Kosuge et al., 2008) and 3 (Lacour et al., 2007).

3.2.3.2. Excitatory amino acid neurotoxicity.

Kainate-induced death takes the form of a strictly region-specific, irreversible, necrotic process that affects the CA3, but not CA1 neurons (Holopainen et al., 2004). However, other authors have observed a massive death of pyramidal neurons in the CA1 and CA3 regions exposed to NMDA. Morphologically, the neuronal death was in this case neither apoptotic nor necrotic but had the hallmarks of autophagy (Borsello et al., 2003). The excitotoxic neuronal injury induced by kainic acid can be dose-dependently attenuated by antioxidants through inhibition of reactive oxygen

species generation, and mitochondrial dysfunction (Kim et al., 2008). Resistance to kainate (and glutamate) is also observed after slice delivery of the HSV-2 anti-apoptotic protein ICP10PK by the replication incompetent virus mutant DeltaRR (Gober et al., 2006; Laing et al., 2008).

3.2.3.3. **Ischemia**. Hippocampal slices are widely used as a model of ischemia-induced neuronal death, given that a brief ischemic insult induces a selective neuronal loss in the CA1 region, which develops in the form of a delayed apoptosis (Finley et al., 2004; Holopainen, 2005; Bali et al., 2007). Activation of caspase 3 was visually illustrated by confocal microscopy, demonstrating a region-selective increase in active caspase 3 signals, which temporally preceded cell death (Cho et al., 2004). Nonetheless, ultrastructural examination of nerve cells in organotypic cultures of rat hippocampus exposed to an anoxic insult, revealed the morphological features typical for both necrosis and apoptosis (Naganska and Matyja, 2001). It was also shown that ionotropic glutamate receptors and glutamate transporters are involved in the necrotic neuronal cell death induced by OGD (Bonde et al., 2005). In addition, experiments in murine organotypic cultures after genetic engineering confirmed the intervention of excitatory amino acid glutamate transporter 2 (EAAT2) in the increased vulnerability to neurodegeneration of CA1 pyramidal neurons (Selkirk et al., 2005). Glutamate neurotoxicity in hippocampal slices is exacerbated by clusterin, a glycoprotein known to be upregulated during tissue involution in response to hormonal changes, injury or other circumstances leading to apoptosis (Hakkoum et al., 2008), and by a deficiency in cellular prion protein (Rangel et al., 2007). Also, a transient exposure to specific pro-inflammatory stimuli can prime hippocampal neurons sus- ceptibility to a subsequent excitotoxic insult (Bernardino et al., 2008). On the other hand, hypothermia was shown to be protective against CA1 and CA3 neuronal cell death induced by OGD (Feiner et al., 2005). Interestingly when hippocampal slices were subjected to hypoxia and subsequently re-oxigenated, the initial phase of apoptosis was followed by stem cell proliferation, neuronal progenitor cell differentiation, and targeted migration to the site of pyramidal neuronal loss (Zhou et al., 2004). Increased neurogenesis in hippocampal slices has also been observed following treatment with the p38 mitogen-activated protein kinase inhibitor SB239063 (Strassburger et al., 2008).

3.2.3.4. **Aβ neurotoxicity**. A number of slice studies have been devoted to the analysis of beta-amyloid peptide (Ab) toxicity aiming to shed more light on the pathogenesis of Alzheimer disease (AD). Treatment of P35 cultures with Ab resulted in a time- and concentration-dependent increase in neuronal injury, whereas immature slices were shown to be resistant (Bruce et al., 1996; Tardito et al., 2007). These observations substantiated the correlation between neuronal susceptibility and aging in the organotypic model. Similarly to what occurs in vivo, plaque-like Ab aggregates can also be observed in slices (Harris-White et al., 1998) and appeared to be enhanced by treatment with transforming growth factor-beta (TGF-b). It was also shown that TGF-b2 can target Ab to neurons in

association with increased ApoE release (Harris-White et al., 2004). In addition, a low-density lipoprotein receptor-related protein (LRP) antagonist, RAP, was able to effectively block TGF-b2-mediated targeting of Aβ to CA1 neurons in both organotypic hippocampal cultures and intact brain infused with Aβ. The utility of hippocampal organotypic slices as a model to study Ab neurotoxicity was confirmed very recently by analysis of Ames dwarf mice. In these animals, characterized by a long lifespan and a marked tolerance to cellular stress, neurons are resistant to Ab-induced tau hyperphosphorylation and changes in apoptosis-regulatory protein levels (Schrag et al., 2008).

3.3. Cerebellum

3.3.1. Main histological features

Cerebellar cultures have been obtained from a relatively wide developmental interval from late embryonic to early or late post- natal animals. Typically, the range goes from E17 to P8. Slicing is associated with a deafferentation of the cerebellar cortex, since the mossy and climbing fibers (the two morphological types of axons reaching the cortex) are cut during the preparatory procedures (Fig. 1C). Efferent connections from the cortex, i.e. the axons of the PNs, may be spared in slices containing the cerebellar nuclei that represent the main synaptic target of these neurons. Roller tube cultures obtained from P0 to P1 mice share many of the features of the adult mouse cerebellum in vivo (Dupont et al., 2006; Lonchamp et al., 2006). In cultures obtained with the interface method, the maturation of PNs was used by some authors as a means to study culture vitality and architecture. It was thus shown that the age of donor animals at the moment of the explants is critical to the subsequent culture development (Fenili and De Boni, 2003). When slices are obtained from P2 mice PNs developed cytotypically, but some were ectopically located; instead when P6 mice were used, PNs develop in an organotypic manner (Fig. 2). The availability of genetically engineered mice in which PNs have been specifically tagged by a reporter gene is advantageous for better culture characterization. Among the genes contributing to the molecular identity of PNs is Purkinje cell protein 2 (Pcp-2). This gene was identified via the cDNA clones PCD5 (Nordquist et al., 1988) and L7 (Oberdick et al., 1988, 1990) and is noteworthy because Pcp-2 mRNA and the 99-amino-acid cytoplasmic protein it encodes have been found only within cerebellar PNs and retinal bipolar neurons (Nordquist et al., 1988; Oberdick et al., 1988). L7GFP mice have been developed where expression of GFP in PNs is driven by the Pcp-2 promoter (Zhang et al., 2001), and we are currently employing these mice in culture experiments (Fig. 2C–E). PNs expressing GFP in these cultures displayed a well-polarized morphology with a single apical dendritic arborization extending across the molecular layer. The distal dendritic branches are covered with spines. In certain instances, it is possible to encounter ectopically placed PNs in IGL. These ectopically placed cells displayed a series of abnormal morphological features such as the presence of multiple primary dendrites, emitting independent dendritic trees (Fig. 2C). An organotypic development of PNs was confirmed after

immunocytochemical staining for 28 kDa calbin- din in cultures obtained from P6-8 mice after 8 DIV. Immunola- beled PNs are initially arranged in a few rows (Fig. 2F), but, after sufficient maturation of the culture is attained, they formed a monolayer, as occurs in vivo (Lossi and Gambino, 2008). In slices devoid of nuclear neurons, a large number of PN axons run back to the PN layer and it was suggested that these cells are extensively self innervated (Dupont et al., 2006).

Plasticity of cerebellar cultures appears to be deeply influenced by the addition, in certain experiments, of DNA synthesis inhibitors in order to prevent glial cell proliferation (Dupont et al., 2006). We currently do not employ these inhibitors because their use would obviously also affect neuronal proliferation, as this may be one of the factors responsible for the reduction (to about one fourth) of the CGC to PN ratio in organotypic cultures where DNA synthesis is blocked. Dupont et al. (2006) used a panel of antisera to immunocytochemically identify the different types of cerebellar neurons in their cultures. These included antibodies against calbindin to label the PNs, Kv3.1b potassium channel to label the CGCs, parvalbumin and glutamic acid decarboxylase (GAD) to label the interneurons. We have also successfully employed a set of markers to this purpose (Fig. 2). Among them, the astrocytic marker glial fibrillary acidic protein (GFAP), and several neuronal markers for some of the neurons which are detected in the developing and mature cerebellar cortex, such as, for example, Zic1 and 2, two zinc finger proteins, and PAX6, a transcription factor, all expressed in CGCs at different developmental stages.

3.3.2. **Neurogenesis and NOND**

No studies have specifically addressed the issue of neurogenesis in cerebellar organotypic slices. This is likely because proliferation, differentiation and migration of CGC precursors have been extensively investigated in vivo (see Altman and Bayer, 1997). Data on cell death mainly regard the PNs and may be summarized as follows. Most PNs in rodent cerebellar organotypic cultures die when the explant is made from P1 to P5, but they survive well when taken before or after these ages (Dupont et al., 2006; Fenili and De Boni, 2003; Ghoumari et al., 2000, 2005). Although quantitative data on PN death during development are still fragmentary, there is increasing evidence suggesting that these neurons, similarly to most other central neurons, undergo PCD (for review, see Lossi and Gambino, 2008). In cultures from the P3 mouse cerebellum, PNs undergo massive apoptosis within the first 36 h in vitro, and display typical ultrastructural features. These include DNA fragmentation, caspase 3 activation, and a strong dependency on expression of the pro-survival factor bcl-2 (Ghoumari et al., 2005). It is of interest to recall here that apoptosis of PNs in P3 cerebellar slices was strongly reduced following elimination of microglia, by a mechanism similar to that reported in vivo (Marin-Teva et al., 2004). In addition, the steroid analog mifepristone protected P3 PNs from death. This effect did not involve the classical steroid nuclear receptors, but was consequent to PN membrane depolarization (Ghoumari et al., 2006). These results suggest

a role of excitatory inputs in PN survival during early post-natal development. In keeping with this suggestion, co-culturing cerebellar slices with glutamatergic inferior olivary neuron preparations made it possible to rescue PNs from apoptosis (Mariani et al., 1991).

Data on NOND in other types of cerebellar neurons, particularly CGCs, are relatively abundant in vivo (Lossi and Gambino, 2008), but largely incomplete in organotypic cultures. There may be several reasons for this paucity of information. First, whereas from P3 onward PNs consistently express calbindin and are thus easily detected by simple, reproducible, immunocytochemical proce- dures, there is a much more complex array of markers for the other cerebellar cortical neurons, strictly related to the stage of explant and the degree of slice maturation in vitro. Second, CGCs are the most numerous cell types in CNS and their counting in slices is made difficult by a series of technical difficulties. Third, rodent CGCs undergo massive apoptosis in vivo in the P5-P10 interval, but also appear to die as a consequence of the explantation. Our studies in P7 murine cerebellar cultures after PI staining demonstrate that there is a massive cell death during the first 3 DIV, with a subsequent stabilization (Fig. 3A and B). Although we have not directly labeled CGCs in these preparations, the remarkably high number and the laminar distribution of PI labeled nuclei strongly suggest that most of them belong to CGCs. In keeping with this suggestion, ultrastructural examination of slices has confirmed the presence of apoptotic CGCs in the granular layers of the forming cerebellar cortex (Fig. 3C and D). Therefore, only in cultures older than 7 DIV does the extent of NOND appear to be safely comparable to the in vivo condition. It is also worth noting that we did not find any difference in the extent and time course of PI staining between cultures grown in the presence or absence of serum. In vivo, we have previously shown that only apoptotic CGCs in the IGL were immunopositive for the cleaved caspase 3 executioner caspase (Lossi et al., 2002b). By biolistic gene delivery in an organotypic slice preparation, we have subsequently been able to confirm these findings and to demonstrate that the absence of caspase 3 activation in the external granular layer (EGL) of the cerebellar cortex was not due to an increased clearance of cleaved caspase 3 immunopositive cells, but rather to occurrence or different mechanisms of apoptosis in the proliferating CGCs that populate the EGL versus the post-mitotic CGCs in IGL (Lossi et al., 2004b).

3.3.3. Experimental cell death

3.3.3.1. **Excitatory amino acid neurotoxicity.** Initial studies under- taken to characterize the type of cell death that follows activation of glutamate receptors demonstrated a different susceptibility of the various cerebellar neurons to excitotoxic insults. Application of NMDA led to necrosis of differentiating CGCs and deep nuclear neurons, whereas kainate led to death of the Golgi cells. Interestingly, the toxic effects of both agonists were prevented if they were dissolved into a solution containing a reduced amount of calcium. AMPA induced

the degeneration of most PNs together with a population of Golgi cells. Three modalities could be discerned: an acute one, in which neurons underwent a rapid necrotic degeneration; a delayed one, in which neurons appeared to be only mildly affected immediately after a 30 min exposure but then underwent a protracted degeneration during the postincuba- tion period (1.5–3 h); and, finally, a slow toxicity, which took place during long (2 h) exposures to AMPA. The two latter mechanisms were likely apoptotic (Garthwaite and Garthwaite, 1986, 1991). Isoflurane (a commonly employed anesthetic) preconditioning reduced the neuronal injury/death that followed glutamate receptor overstimulation. This neuroprotection may be protein kinase C (PKC)- and nitric oxide synthase (NOS)-dependent (Zheng and Zuo, 2005).

3.3.3.2. **Ischemia.** OGD was responsible for significant PN injury and death, an effect reverted by preconditioning with isoflurane (Zheng and Zuo, 2003, 2005) or morphine (Lim et al., 2004). Also, hypothermic preconditioning induces an acute phase of neuro- protection in OGD. This neuroprotection depends on activation of adenosine A1 receptors, KATP channels, Ras, and inducible NOS (Yuan et al., 2004, 2006). The consequences of cerebellar ischemia on the electrical activity of PNs have also been investigated. In one study the dominant cause of the electrophysiological dysfunction of PNs was linked to an activation of AMPA receptors. The glutamate activating these receptors is released both by exocytosis (at early times) and by reversal of a glutamate transporter (Hamann et al., 2005). By two-photon cellular imaging, the ischemia-induced functional deterioration of PNs was shown to be accompanied by cytoplasm Ca^{2+} rise and prevented by BAPTA infusion (Zhao et al., 2008b). Therefore, ischemia destabilizes the spike encoding of PNs by raising cytoplasm Ca^{2+} without a need for glutamate, which is then responsible for their excitotoxic death.

3.4. Retina

Organotypic retinal cultures have been prepared from explants and/or slices (Fig. 1D). In both cases a normal cytoarchitectural development was achieved (Hofmann, 2005). Retinae are usually dissected free from the retinal pigment epithelium (RPE) during culture preparation. Under these conditions, development in vitro was very similar to that in vivo, except for the lack of differentiation of the photoreceptor outer segments that was found to be dependent on the contact with the RPE. In rodent retina, the majority of cells differentiate during the first 2 weeks after birth. Therefore, retinae from P2 rats that are often used for explantation are highly immature, and have a tendency to display a reduced thickness, particularly at the level of the ganglion cell layer that tends to disappear as a consequence of the retinal ganglion cell (RGC) axotomy (Caffe et al., 1989; Pinzon-Duarte et al., 2004, 2000). A novel organotypic culture method of P0-14 mouse retina explants has recently being introduced. Retinal whole-mounts were cultivated on poly-D-lysine/laminin coated coverslips. After 7 DIV, explants were treated with varying concentrations of L- glutamate and cell death was

accessed with TUNEL and immunocytochemistry. In contrast to previously published meth- ods using slice or floating whole-mount cultures, the ex vivo culture system presented here combines accessibility to experi- mental manipulation, and adherence of whole-mount cultures to a substrate with a significant preservation of retinal cell types, numbers and morphology (Xin et al., 2007). Even more recently, retinae explanted from adult rats were cultured in serum-free medium (B27/N2) and tissue viability was assessed by gross morphology, PI uptake, cell survival quantifica- tion, activated caspase-3 expression, and immunohistochemistry. Explants were viable for at least 17 DIV and expressed several markers of neuronal differentiation. They also mimicked in vivo glial reactivity to transplantation (Johnson and Martin, 2008). Additional new methods for culturing the adult mammalian retina were also developed (Kaempf et al., 2008b; Kobuch et al., 2008). In this case the entire adult porcine retina was cultivated together with the RPE. After 3 DIV, a limited nuclear loss and a significant reduction in apoptotic cells was observed in retina-RPE cultures compared to retinal cultures alone, in parallel with decreased Mü ller cell hypertrophy. This approach has proved useful in showing that bevacizumab, a molecule used for treatment of exudative age-related macular degeneration, is well tolerated by RGCs and photoreceptors even at concentrations fivefold higher than those used clinically (Kaempf et al., 2008a).

3.5. Others

Spinal cord slice cultures have frequently been employed as an in vitro model of amyotrophic lateral sclerosis (ALS). P8 rat slices can be grown in culture for several weeks and retain good architecture of the grey matter. In these slices there is an apparent reduction in the number of ventral horn motorneurons, likely as a consequence of axotomy. Chronic inhibition of glutamate trans- porters led to slow motor neuron degeneration that could be blocked by non-NMDA receptor antagonists (Corse et al., 1999; Kaal et al., 2000). Apparently, motor neurons in the slice preparation also have the adult GluR2 subunit constellation of their AMPA receptors, as NMDA receptor antagonists were not effective in protecting against motor neuron death (Rothstein et al., 1995). By chronically exposing slices to slightly increased concentrations of glutamate, leading to chronic Ca^{2+} overload and shortage of ATP, the continuous drain of energy made motor neurons more vulnerable to glutamate toxicity (Albin and Green- amyre, 1992). This model mimicked one of the most characteristic traits of ALS, i.e. the selective motor neuron vulnerability. Selective motor neuron death appeared to be apoptotic, and could be totally blocked by anti-oxidants, and partially by an AMPA inhibitor, 6- cyano-7-nitroquinooxaline-2,3-dione (CNQX) (Kaal et al., 2000). Protection against glutamate-induced motor neuron death in organotypic spinal cord cultures is also offered by huperzine A, citicoline, and vascular endothelial growth factor (VEGF), three molecules with neuroprotective abilities (Hemendinger et al., 2008; Matyja et al., 2008; Tolosa et al., 2008). Interestingly, BDNF was not neuroprotective in these cultures,

although spinal motor neurons express TrkB receptors (Yan et al., 1994). In view of these results, the organotypic glutamate toxicity model may provide a useful predictive preclinical model for ALS in forecasting the outcome of these trials. In keeping with this possibility, spinal cord organotypic cultures have recently been used as a tool to test the response of motor neurons to serum IgGs from ALS patients (Li et al., 2008). Finally, neurodegeneration in dopaminergic neurons (a well known cause of Parkinson's disease in vivo) has been successfully reproduced in striatal slices (Testa et al., 2005).

3.6. Glial cells in organotypic cultures

Astrocytes represent the most numerous population of non- neuronal cells in the brain, but microglia represent approximately 15% of the adult brain glia population. A third major type of glia is represented by the oligodendrocytes that produce the myelin sheets of central axons. The interplay between these glial cells and the neurons has critical roles during normal development as well as in disease, and this issue is beginning to be addressed in slice studies.

3.6.1. Astrocytes

As mentioned previously, if cultures are not maintained in the presence of DNA inhibitors, a layer of astrocytes becomes apparent on the surface of slices even in the absence of activating stimuli, and proliferation of astrocytes on the surface of cultures obtained with the air interface method appears to be a common feature of this type of preparation (Fig. 2G). Immediately following explanta- tion in these cultures not only is there an extensive proliferation of astrocytes (mostly reactive type II), but also of microglia and fibroblastic cells. This leads to the formation of a type II astrocytic glial cover on the top of the sectioned surface, whereas the majority of type I astrocytes and fibroblasts are confined to outgrowth zones at the substratum/culture interface. The inhibi- tion of astrocytic proliferation may have consequences in the cytoarchitectonic rearrangement that follows the initial phase of explant growth in culture. For example, if the astrocytic proliferation is inhibited, persistence of long-distance growing of tyrosine hydroxylase-positive nerve fibers is seen in ventral mesencephalic cultures (Af Bjerké n et al., 2008). Astrocytes in slices are capable of responding to an array of experimental challenges as in vivo. For example, a brief application of NMDA or induction of endoplasmic reticulum stress activated hippocampal astrocytes (Lacour et al., 2007; Pizzi et al., 2004). Outgrowth, proliferation and migration of these glial cells were enhanced when slices were treated with inhibitors of proteoglycan synthesis (Berglö f et al., 2008), or with TNFa (Marschinke and Strö mberg, 2008). In keeping with this latter finding, inhibitors of the Rho-kinase, which down regulate TNFa, decreased the number of reactive astrocytes (as well as retinal Mueller cells, and microglia) and GFAP immunoreactivity in retina (Tura et al., 2008).

3.6.2. Microglia

Brain microglia comes from both infiltrating blood monocytes and resident

progenitor cells. Evidence supporting the presence of endogenous proliferating microglia has been provided in slices, by challenge with several stimuli inducing proliferation. For example, local proliferation (activation) of microglia in response to injury was described in neocortical slices (Eliason et al., 2002), and to TNFa in mesencephalic slices (Marschinke and Strö mberg, 2008). Microglial cells in hippocampal slices respond normally to transient application of specific stimuli mimicking inflammation, with a pronounced proliferation followed, in certain circum- stances, by apoptotic-like death (Laskowski et al., 2007; Bernar- dino et al., 2008). In keeping with this observation, anti- inflammatory treatment with the p38 mitogen-activated protein

kinase inhibitor SB239063 decreases the number of activated microglia (Strassburger et al., 2008). Very recently, an assay system was developed to study in vitro prion toxicity. In this system, by using cerebellar slices from transgenic mice where microglial release of nitrite, proinflammatory cytokines and chemokines was abolished, it was demonstrated that microglial activation represents an efficacious defensive reaction against prion diseases (Falsig et al., 2008).

3.6.3. Oligodendrocytes

Culture models have proved useful in research on neuronal demyelination and oligodendrocyte degeneration. A brief applica- tion of NMDA to organotypic hippocampal slices induced oligodendrocyte degeneration secondary to an astrocytic reponse. Treatment with a chimeric derivative of interleukin-6 (IL-6) and soluble IL-6 receptor resulted in preserving myelin basic protein (MBP) production (Pizzi et al., 2004). This result is consistent with the protective role of IL-6 shown in vivo. Cerebellar slices demonstrated significant myelination after 1 week in culture. Treatment of the cultures at 7 DIV with the bioactive lipid lysolecithin produced marked demyelination, as determined by immunostaining for several myelin components. After a transient demyelinating insult with lysolecithin, cultures recovered with de novo oligodendrocyte differentiation (Birgbauer et al., 2004). No evidence for oligodendrocyte proliferation has been detected in slices (Cho et al., 2007). In other experiments it was shown that hypoglycemia inhibits oligodendrocyte develop- ment and myelination, and triggers apoptotic cell death in oligodendrocyte precursor cells (Yan and Rivkees, 2006).

4. Conclusion

Brain slices represent very useful tools for analysis of cell proliferation and death in neurons and glia. Slices can be easily obtained from several areas of CNS, and novel techniques are still developed to maintain in vitro not only embryonic or early post- natal tissue, but also the adult brain. Changes in neuronal circuitry and synaptic plasticity that follow the preparation of explants have mostly been characterized in detail, and can be fundamentally related to degeneration of the projection pathways that are inevitably severed while slicing the brain. Neurogenesis and NOND not only can be monitored in acute slices, but also in organotypic cultures that can be maintained alive and amenable to experi- ments for several weeks. To this purpose, it will be crucial in the future to develop additional specific fluorescent probes that allow for dynamically tracking individual cells over time. Organotypic slices have also proven useful to the study of amino acid excitotoxicity, ischemia, and neurodegenerative diseases. Allto- gether these properties make them an attractive alternative/ complement to experimentation in vivo and single cell studies.

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