Neurochemical Research, Vol. 30, No. 12, December 2005 (© 2005), pp. 1521–1528
DOI: 10.1007/s11064-005-8829-5

Organotypic Hippocampal Slice Cultures: A Model System to Study Basic Cellular and Molecular Mechanisms of Neuronal Cell Death, Neuroprotection, and Synaptic Plasticity*

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(Accepted September 12, 2005)

The hippocampus has become one of the most extensively studied areas of the mammalian brain, and its proper function is of utmost importance, particularly for learning and memory. The hippocampus is the most susceptible brain region for damage, and its impaired function has been documented in many human brain diseases, e.g. hypoxia, ischemia, and epilepsy regardless of the age of the affected patients. In addition to experimental *in vivo* models of these disorders, the investigation of basic anatomical, physiological, and molecular aspects requires an adequate experimental *in vitro* model, which should meet the requirements for well-preserved representation of various cell types, and functional information processing properties in the hippocampus. In this review, the characteristics of organotypic hippocampal slice cultures (OHCs) together with the main differences between the *in vivo* and *in vitro* preparations are first briefly outlined. Thereafter, the use of OHCs in studies focusing on neuron cell death and synaptic plasticity is discussed.

KEY WORDS: Excitotoxicity; hippocampal slice cultures; nerve cell death; neuroprotection; plasticity.

CELLULAR AND FUNCTIONAL ORGANIZATION OF ORGANOTYPIC HIPPOCAMPAL SLICE CULTURES

The hippocampus is characterized by a distinct laminated structure, stratum pyramidale and stratum granulosum, composed of the pyramidal CA1, CA2, and CA3 neurons, and the dentate gyrus (DG) granule cells, respectively (1,2). Functionally, the excitation enters the DG granule cell dendrites via the perforant pathway, runs along the granule cell axons, the mossy fibers (MF), exciting CA3 pyramidal cell dendrites, which convey the information through the Schaffer collaterals to CA1 neurons, and the excitation leaves the hippocampus through the alveus and the fimbria. In principle, this trisynaptic pathway is functionally similar both *in vitro* and *in vivo*. It is excitatory in nature, and uses glutamate as its primary neurotransmitter (3), while the final output of this excitatory loop is modulated and fine-tuned by the inhibitory GABAergic interneurons at different steps (4).

Hippocampal slice cultures are in general made according to the method published by Stoppini et al. (5). Cultures are usually prepared from 6–7-day-old (P6–7) rats, although both younger and older rats (from P4 up to P30) have been applied, and slices can be maintained in culture conditions from one week

* Special issue dedicated to Dr. Simo S. Oja.

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0364-3190/05/1200–1521/0 © 2005 Springer Science+Business Media, Inc.

up to 1–2 months (6–10). When prepared from P6–7 rats, dentate granule cells are still proliferating, while the other main neuronal cell types, e.g. CA1–CA3 pyramidal neurons, and interneurons are at postmitotic stage (11,12). In culture conditions, the laminated structure as well as the main characteristic morphological organization of the hippocampus is well-preserved, and the maturation of different cell types, synaptic contacts, and receptor expression resembles that seen *in vivo* (13–16). By comparing immature (7 DIV, days *in vitro*) and mature (around 21 DIV onwards) OHCs, developmental differences in death mechanisms and plasticity of the hippocampal circuitry can be elucidated.

There are, however, some differences between the *in vivo* and *in vitro* hippocampal tissue, which may be of functional importance. For example, there is an increase in proximal dendrites and total dendritic length of dentate granule cells both *in vivo* and *in vitro* up to 15 DIV, whereas at 20 DIV, these parameters as well as the branching index (free dendrites/stem dendrite), and the number of spines on granule cell dendrites were significantly lower *in vitro* compared with their *in vivo* counterparts (14). Lack of extrinsic afferents rather than deterioration of granule cells *per se* were proposed to be the main factor contributing to this reorganization pattern of dendritic arborization. Another important feature in cultures is the collateral sprouting of dentate granule cell axons, the MFs, which progressively increases in the DG molecular layers during the early *in vitro* maturation of hippocampal slices resulting in intense sprouting by 3 weeks (17). Although the main excitatory loop from dentate granule cells to CA3, and further to CA1, is preserved, this reorganization contributes to the progressively increased excitatory activity of granule cells during the culture time (8,18,19). In spite of these major differences in the properties between the *in vivo* and *in vitro* hippocampus, cultured slices nevertheless meet well the criteria for an excellent *in vitro* model to elucidate cellular and molecular basics of the normal hippocampal function and pathology in response to external disturbances.

HIPPOCAMPAL SLICE CULTURES AS A MODEL FOR EXCITOTOXIC NEURONAL DAMAGE

Current evidence suggest that neuronal death can occur through necrosis, apoptosis, autophagic and endocytic processes, or through a combination of these mechanisms (20). Neuronal cell death caused by pathological overactivation of glutamate receptors (excitotoxicity), is a contributing factor in a number of central nervous system insults and disorders, including hypoxia, ischemia, and epileptic seizures (21–25). Currently, it is considered that excitotoxic neuronal death can be mediated through any of these main mechanisms alone or combined, and various intracellular cascades are simultaneously, or sequentially, involved. Studies using either immature or mature OHCs have increasingly been used to elucidate these basic mechanisms of neuron cell death.

In OHCs cultured for 3–4 weeks, treatment with different glutamate receptor agonists for 2 days has induced neuronal death with different potency. The lowest EC_{50} value has been detected for (*RS*)-2amino-3-hydroxy-5-methyl-4-isoxazole (AMPA, 3.7 μM), followed by *N*-methyl-D-aspartic acid (NMDA, 11 μM) and kainic acid (KA, 13 μM), the less effective being the KA receptor selective agonist (*RS*)-2amino-3-(3-hydroxy-5-*tert*-butylisoxazol-4-yl) propionic acid (ATPA 33 μM) (26). The most vulnerable cell type seems to be the CA3 pyramidal neurons, although higher agonist concentrations and prolonged treatment lead to a more wide-spread damage including CA1 neurons and DG granule cells (9,26–29). Moreover, there seems to be a developmental shift towards increasing sensitivity to KA toxicity during the *in vitro* maturation of slices, whereas this has not been observed for the NMDA-induced toxicity (9,28).

KA treatment of OHCs has also been used as an *in vitro* model to study mechanisms of status epilepticus (SE)-induced neuronal damage and epileptogenesis, since KA treatment of hippocampal slices induces region-specific neuronal death and reorganization of the hippocampal circuitry (9,19,29), features similar to those detected in experimental epileptic models of SE and during epileptogenesis (30–33). The 48-h KA (6–7 μM) treatment has resulted after a prolonged culture time (40–60 days) in loss of CA3a/b pyramidal cells, slight decrease in the number of granule cells, robust granule cell axonal sprouting, epileptiform bursts, and spontaneous excitatory postsynaptic currents without any changes in inhibitory GABAergic function (34). Although similar changes, but less robust, were verified in cultures grown in normal culture medium, it was concluded that in addition to recurrent granule cell network after MF sprouting, synaptic connections from CA1, and potentially also from CA3 to granule cells in KA-treated cultures, can all contribute to granule cell hyperexcitability.

A representative example of a region - specific KA-induced nerve cell death is shown in Fig. 1. Hippocampal slice cultures were prepared from 4-day-old rats and cultured for 7 days as recently described (9). KA (5 μM) was added for 12 h, and after the fixation, slices were stained with Fluoro-Jade B as recently described (29). Fluoro-Jade B stains the degenerating neurons regardless the mechanisms by which a nerve cell dies. The images were documented with a confocal microscopy system acquired at 2 μm intervals, analyzed, and the image of the whole hippocampus was reconstructed.

The caspase family of enzymes plays an important role in apoptosis (35,36). The activation of caspase pathway starts with caspase-9, and once activated, it cleaves a 32-kDa inactive caspase-3 into a 17–18-kDa active caspase-3 (36,37). In addition to this intrinsic mitochondrial pathway of caspase activation, a second, extrinsic pathway activates caspase-8, which then directly activates caspase-3 (38). The contribution of apoptosis in KA-induced neuronal death was verified in 20–25 DIV, mature OHCs, in which a rapid induction of bax, a pro-apoptotic factor, collapse of mitochondrial membrane potential, release of cytochrome c from mitochondria, and activation of caspase-3 suggest the activation of multiple apoptotic pathways in response to KA treatment (39). At variance with this, in 7 DIV, immature OHCs, the robust time-dependent KA-induced CA3 neuronal damage showed no signs of caspase-3 activation, and no cleavage of poly(ADP ribose)polymerase (PARP), a 116-kDA nuclear DNA repair enzyme, was detected, suggesting that KA treatment resulted in non-apoptotic neuronal damage (29). This was verified with electron microscopic analysis, which revealed changes in CA3a/b neurons typical for necrotic process, i.e. pycnotic neuronal cell bodies with swollen intracellular structures, particularly mitochondria, and devoid of nuclear fragmentation. These studies in OHCs are strikingly similar to *in vivo* studies, which indicate fundamental differences between the immature and mature hippocampus in response to trauma, hypoxia--ischemia, and excitotoxic lesion (40–43).

In addition to necrotic and apoptotic glutamate agonist-induced nerve cell death, a recent study in OHCs revealed that autophagy and endocytosis can also be involved in death process. The application of NMDA (100 μM) to OHCs resulted in fulminant death of CA1 and CA3 pyramidal neurons within 2 h (44). Dying neurons exhibited autophagic and endocytic features, and both the neuronal death, and the associated autophagy and endocytosis, were prevented with a JNK-binding peptide inhibitor (D-JNKI1) (44). Consequently, the death was suggested to be under the control of the c-Jun *N*-terminal kinase pathway. The endocytosis occurred in most neurons several hours before they died, suggesting that it was initiated by events upstream of the death effector mechanisms. This study strongly suggests that autophagy is an additional, novel mechanism involved in neuronal death in response to excitotoxic stimulus in the hippocampus (44).

HIPPOCAMPAL SLICE CULTURES AS A MODEL OF ISCHEMIA-INDUCED NEURONAL DEATH

In the rodent models of transient ischemia, the hippocampus has been shown to be one of the most susceptible brain regions (21). A brief insult induces a selective neuronal death in the CA1 region, which develops over the following 2–4 days after ischemia, described as a delayed mode of neuronal death (45). Although excessive release of glutamate and subsequent increase in the intracellular calcium are considered to be of importance at the acute phase of neuronal damage (46), recent studies strongly suggest the involvement of apoptosis in the ischemia-induced delayed neuronal death (47,48). In OHCs, oxygen–glucose deprivation (OGD) is widely used as an *in vitro* model of ischemia. In this model, OGD induces neuronal death within 4 h in the CA1 region, and the damage extends to the CA3 regions during the following 72 h (49). Moreover, experimental OGD elicited extensive increases in the release of GABA and aspartate, and lead to a time-dependent activation of caspase-3. In OHCs prepared exceptionally from 20- to 30-day-old rats and cultured for 1 week, lactic acidosis was identified as a contributor to excitotoxic CA1 and CA3 neuronal loss, the effect being at least partly mediated through the activation of both NMDA and AMPA receptors (10).

The expression and release of proinflammatory cytokine tumor necrosis factor-α (TNFα), which is synthesized and released by astrocytes, microglia, and some neurons, are rapidly increased in various pathological conditions, such as trauma, ischemia, and inflammatory diseases (50). In rat organotypic hippocampal-entorhinal cortex slice cultures, TNFα has enhanced glutamate neurotoxicity in the CA3 and CA1 regions by directly inhibiting glutamate uptake

this effect being prevented by NFκB antagonists (51). These results suggest that NFκB antagonists could served as therapeutic agents in diseases associated with oxidative stress, combined with increased levels of TNFα and glutamate.

Cytoskeletal integrity of nerve cells is crucial for their morphology, survival, and regulation of organelle transport (52,53), and any disturbance in the intracellular filament structure could have a deleterious effect on neuronal viability. In ischemia, the increased intracellular Ca^{2+} levels are proposed to lead to overactivation of intracellular calcium-activated proteases, such as calpains, which could contribute to cytoskeletal degradation and enhance cell death (54). Using the *in vitro* OHD model of ischemia, rapid breakdown of spektrin, an indicator of activation of calpain protease, was detected in CA1 and CA3 pyramidal neurons of OHCs (55). The calpain inhibitor MDL-28170, when added after the ischaemic insult, had a clear neuroprotective effect (55). In addition to neuronal death, degradation of neurofilament (NF) proteins, the major class of intermediate filaments (52) was verified in OHCs after the KA treatment (5 μM, 48 h) combined with a 2-days recovery period in normal medium. The effects were more pronounced in older than in younger cultures (9). Although the contribution of cytoskeletal degradation to nerve cell death is poorly understood, the downstream targets in excitotoxic cascade could involve calpains.

NEUROPROTECTION AND SYNAPTIC PLASTICITY IN ORGANOTYPIC HIPPOCAMPAL SLICE CULTURES

In addition to application of OHCs to neuronal death studies, these cultures provide an excellent *in vitro* model system to study physiological factors and pharmacological compounds contributing to neuronal survival and synaptic plasticity. By varying the culture time, and the age of animals for the slice preparation, age-dependent differences in neuronal vulnerability and survival can be analyzed. It has recently been shown that the OGD-induced increase in intracellular Ca^{2+} in CA3 neuronal soma is caused by a concomitant activation of NMDA receptors, Na^{+}/Ca^{2+} exchangers, and Na^{+} channels (56). Neuroprotection was achieved by blockade of NMDA receptors and plasma membrane Na^{+}/Ca^{2+} exchangers, both contributing to reduced Ca^{2+} entry into neuronal soma, while blockade of AMPA/KA receptors and mitochondrial Na^{+}/Ca^{2+} exchangers reduced Na^{+} entry at the dendritic level, and this seemed to enhance neuronal survival (56,57). Moreover, diazepam, when applied shortly after OGD treatment in hippocampal cultures, decreased neuronal excitability and prevented the activation of downstream cell death mechanisms (58).

In addition to calpain-mediated degradation of cytoskeletal proteins in response to ischemic and excitotoxic insult, calpain activation has also been connected to neuronal plasticity. For example, prolonged treatment of OCHs with positive modulators of AMPA receptors was accompanied by increased calcium-dependent calpain activation and spektrin degradation (57), changes suggested to be associated with long-term potentiation (LTP). It was proposed that treatment with AMPA receptor modulators could result in synaptic reorganization similar to that in synaptic plasticity during the establishment of LTP and/or long-term depression (LTD) in hippocampal synapses. Moreover, a brief (3 h) treatment of OHCs with NMDA (100 μM) resulted in the NMDA receptor-mediated activation of calpain and complex modifications of AMPA receptor properties suggesting an effect on synaptic plasticity (59).

Recent evidence suggest that activation of group I metabotropic glutamate receptors (mGluRs) could function as intrinsic physiological regulators of nerve cell susceptibility to injury, and thus be crucial for neuroprotection (60), particularily against ischemic nerve cell death (61,62). In accordance with these studies, the involvement of mGluR1 in neuroprotection against NMDA-induced cell death has recently been demonstrated in OHCs, the putative mechanism being mediated through changes in NMDA receptor/channel function (63).

An interesting question is whether or not antiepileptic drugs, besides inhibiting seizure occurrence, have any neuroprotective effect. It has recently been shown that that newer antiepileptic drugs, such as carbamazepine, lamotrigine, tiababine, and oxcarbazepine had significant neuroprotective effect in OHCs exposed to OHD, a model of ischemia (64). The mechanisms of action were proposed to be mediated through modulation of GABAergic inhibition, and blockade of Na^{+} or Ca^{2+} channels. Also $GABA_A$ receptor agonist THIP (4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridine-3-ol) can protect CA1 and CA3 pyramidal neurons against NMDA-induced excitotoxicity suggesting that enhancement of GABAergic activation is neuroptotective (65).

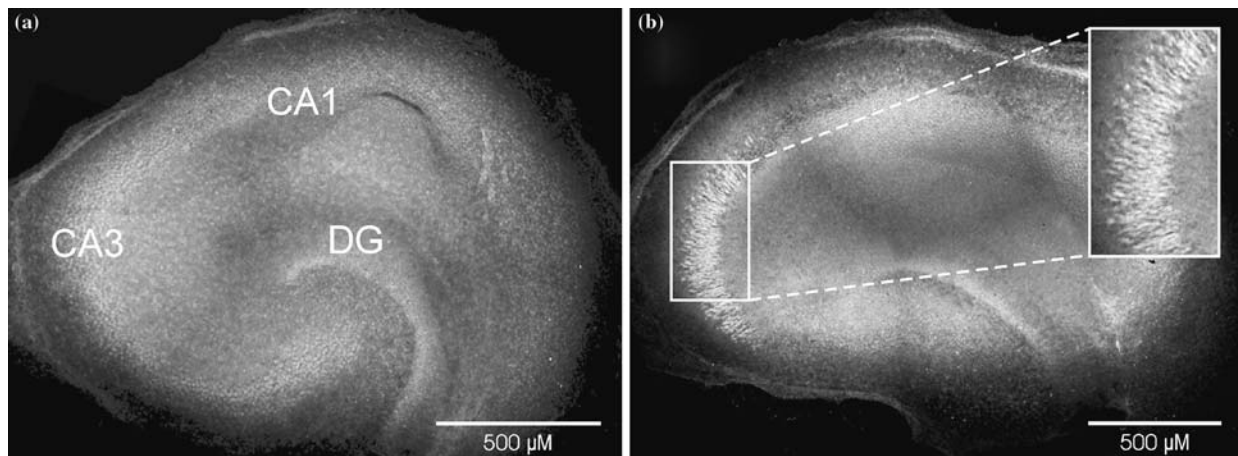

Fig. 1. The KA-induced nerve cell damage in the CA3a/b region of the cultured hippocampal slices. In control cultures, no Fluoro-Jade B-stained neurons were detected (a) indicating good survival of neurons in culture conditions, whereas massive staining was detected in the CA3 pyramidal cell regions in the KA-treated culture (b). The main neuronal cell layers, the CA1 and CA3 pyramidal cell layers, and dentate gyrus (DG) containing granule cells are indicated. The scale bar is 500 μm, as indicated.

PROLIFERATION OF NERVE CELLS IN SLICE CULTURES: A MODEL SYSTEM TO STUDY NEURONAL MATURATION AND PLASTICITY

The importance of nerve growth factors, especially brain-derived neurotrophic factor (BDNF) in the regulation of neuronal survival and plastic changes in morphology and function has been increasingly studied during the recent years. For example, BDNF has been suggested to contribute in MF sprouting and hippocampal reorganization during experimental epileptogenesis in rats (66) and in OHCs (67). Recently, the mechanisms of action of BDNF in seizure-induced synaptic plasticity were studied in OHCs, in which dentate granule cells were transfected with BDNF or nerve growth factor (NGF) by using a particle-mediated gene transfer. The increased expression of BDNF significantly enhanced formation of axonal branching and basal dendrites, and these structural changes were prevented by the tyrosine kinase inhibitor K252a (68). This study indicated that BDNF by activating its receptor TrkB within dentate granule cells was sufficient to induce these morphological changes in OHCs. Of interest was that the most striking morphological effects of BDNF were detected in granule cells near the margin of the hilus and the granule cell layer, without any effect on CA1 and CA3 pyramidal cells.

Hippocampal slice cultures also provide a novel, versatile model system to study neurogenesis, and the anatomical and physiological integration of newly generated neurons into the hippocampal network. After 2 weeks in culture, double immunostaining with the mitotic marker BrdU and cell type-specific markers have revealed persistent proliferation of various cell types (69). The new neurons appeared in a subgranular germinal zone of the DG, and the regional distribution of the newly born granule cells closely resembled that observed *in vivo* in the adult hippocampus (70,71). Neurogenesis was modulated by different factors added to OHC culture medium. For example, the rapid and complete inhibition of neurogenesis was achieved in the presence of serum, while strong overall cell proliferation (mainly glial cells) was induced by a chronic application of the epidermal growth factor (EGF), although its effect on neurogenesis was optimal in serum-free conditions (69). In accordance with this, also another recent study suggests that dentate granule cells in OHCs intrinsically retain spontaneous proliferative abilities throughout the 4-week culture period (72) (Fig. 1).

In summary, OHCs is an excellent, well-controlled model system to study cellular and molecular mechanisms of neuronal death, and to reveal pharmacological tools for neuroprotection. By choosing the optimal *in vitro* age of cultures, developmental aspects of vulnerability and plasticity related changes give additional insight in processes involved in recovery after the insult. Moreover, OHCs provide a novel, powerful tool to study neurogenesis, and clarify the functional, morphological and pharmacological properties of newly born neurons in their native environment.

ACKNOWLEDGMENTS

The contribution of Tiina-Kaisa Kukko-Lukjanov, M.Sc. for providing me with the images of the hippocampus is gratefully acknowledged, as well as the financial support of the Special State Grant for Clinical Research (EVO), and Arvo and Lea Ylppö Foundation.

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