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- (71) **Applicants:** INSTITUT NATIONAL DE LA SANTE ET DE LA RECHERCHE MEDICALE (INSERM) [FR/FR]; 101 rue de Tolbiac, F-75013 Paris (FR). UNIVERSITE PIERRE ET MARIE CURIE (PARIS 6) [FR/FR]; 4 place Jussieu, F-PARIS 75005 (FR).
- (72) **Inventor:** EUGENE, Emmanuel; U739, 105 Bd de l'hôpital, F-75013 Paris (FR).
- (74) **Agent:** LE COUPANEC, Pascale; Nony, 3 rue de Penthièvre, F-75008 Paris (FR).

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(54) **Title:** NOVEL CULTURE MEDIUM FOR IN VITRO ORGANOTYPIC CULTURE OF AN ADULT BRAIN TISSUE SAMPLE

(57) **Abstract:** Nutrient medium of defined composition for the in vitro organotypic culture of an adult brain tissue sample, said nutrient medium comprising (i) one or more pH buffering system(s); (ii) one or more inorganic salt(s); (iii) one or more trace element(s); (iv) one or more free amino acid(s); (v) one or more vitamin(s); (vi) one or more hormone(s); (vii) one or more carbon/energy source(s); wherein said nutrient medium presents an osmolarity of from about 290 to about 330 mOsm, preferably of from about 300 to about 320 mOsm, most preferably of about 310 mOsm; wherein said nutrient medium has a pH of from about 7.0 to about 7.4, preferably of from about 7.1 to about 7.3, most preferably of about 7.2; and wherein said nutrient medium presents a glutamine content and a glutamic acid content of from about 1 μ M to about 250 μ M, preferably of from about 1 to about 150 μ M, most preferably of from about 5 to about 20 μ M.



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Novel culture medium for *in vitro* organotypic culture of an adult brain tissue sample

FIELD OF THE INVENTION

The present invention relates to a nutrient medium that enables to maintain the survival of a multicellular tissue sample for several weeks.

5 More particularly, the present invention relates to a nutrient medium of defined composition for the *in vitro* organotypic culture of an adult brain tissue sample.

BACKGROUND OF THE INVENTION

Epilepsy is a widespread neurological disease in Europe affecting about
10 6 million people. This disease actually includes a set of conditions of the nervous system leading to an epileptic syndrome, for which a hypersynchronous neuronal discharge tends to generalize from a local area in the cortex. This syndrome can be either primary or resulting from a behavioural disorder, for example alcoholism, from a physiological disorder, for example catamenial epilepsy, or from a disease such as a tumor or an
15 autoimmune disease.

75-80% of patients are treated successfully with available pharmacological treatment, for example using at least one of the following drug: carbamazepine, sodium valproate, lamotrigine, phenytoin, oxcarbazepine, ethosuximide, gabapentin, levetiracetam, tiagabine, topiramate, vigabatrin, phenobarbital, primidone and clonazepam. The
20 remaining 20-25% of patients have, however, developed drug-resistance towards conventional treatments.

However, due to a lack of a reliable *in vitro* model of epileptic brain tissue, numerous research programs for assessing efficient therapy for drug-resistant epileptic individuals cannot be conducted.

25 Organotypic culture medium was reported to be useful for various types of research and/or clinical studies on human brain diseases. A plurality of research works have been conducted in the past, with the view of designing improved culture medium compositions for culturing

Survival and maintenance of brain slices from young animals in organotypic
30 culture was successful for several weeks in roller-tube cultures (Gähwiler (1988); Gähwiler *et al.* (1997)). A simplified procedure based on a static culture medium-air interface was later introduced by Stoppini *et al.* (1991).

Ideally, in organotypic cultures, cellular identity and spatial relations between cells shall be maintained throughout the time period of experimental use thereof. To date, organotypic culture is mostly successful with perinatal tissue, for example from postnatal days 0-7 in rodents (Gähwiler (1988); Stoppini *et al.* (1991)) or prenatal human tissue
5 (Hansen *et al.* (2010)). In older tissue, neuronal survival is easily compromised (Gähwiler *et al.* (1997)), and cellular migration is not over in younger tissue.

An illustrative attempt to design optimal culture medium compositions for brain-derived tissue samples is disclosed in US patent application 2005/208028. This document relates to a liquid aqueous medium that improve neural cell viability in brain or
10 spinal cord tissue after brain or spinal cord injury or surgery. This medium has a lower osmolarity as compared to the osmolarity encountered in body fluids and is essentially free of ferrous sulphate, glutamate and aspartate.

In most cases, organotypic culture medium compositions known in the art comprise undefined animal compounds, such as compounds comprised in animal serum or
15 the like (See notably Stoppini *et al.* (1991), Jung *et al.* (2002), Chaichana *et al.* (2007)). However, serum may be detrimental when culturing adult tissues. Indeed, when brain tissue is in contact of serum in vivo following a breach of the blood-brain barrier, for example due to an infection, a trauma, or a disease, may elicit inflammatory responses and potentially apoptosis.

Attempts to maintain tissue from adult non-human animals (Wilhelmi *et al.* (2002); Kleinberger-Doron and Schramm (1990)) or from adult human origin (Jung *et al.* (2002); Chaichana *et al.* (2007); Gonzalez-Martinez *et al.* (2007)) have been moderately
20 successful in terms of cellular survival. However, in these attempts, it is not clear that electrical activity is maintained. In both physiological and pathological contexts, an appropriate activity may be a crucial index of successful culture conditions.

Hence, another challenge of in vitro culture of brain tissue sample from epileptic individuals is to manage maintaining electrophysiological activities of the cortex sample. Indeed, the aim is to monitor the electric activity taking place during the induced convulsions as well as the electric activity in between these convulsions, which is also
25 referred to as interictal activity. As the interictal discharges are the most frequent electrical activity that experience an epileptic individual, this specific electrical activity represents a

signature of the epileptic condition. Furthermore specialized physicians often take advantage of this interictal electrical activity to diagnose epilepsy.

Hence, the design of improvements to in vitro culture conditions of brain tissue samples originating from epileptic individuals are aimed at providing an efficient and realistic in vitro model for studying the epileptic condition, notably with the view of implementing new therapeutic strategies.

Hence, there is a need to provide organotypic culture medium of defined composition, especially for the in vitro culture of brain-derived tissue samples.

There is also a need to provide organotypic culture medium for in vitro culture of adult tissues, especially brain-derived adult tissue samples.

There is still a need to provide organotypic culture medium able to provide survival of all the cellular types within the adult tissues, especially brain-derived adult tissue samples.

There is yet a need to provide organotypic culture medium able to provide conditions of culture that are the closest from the physiological conditions, i.e. the conditions that cells experience in situ within the animal body.

There is also need to provide organotypic culture medium able to maintain the electrophysiological activities of the cells, especially able to maintain the physiological conditions of the various kinds of cells that are contained in a brain-derived tissues sample.

SUMMARY OF THE INVENTION

A first aspect of the present invention relates to a nutrient medium of defined composition for the in vitro organotypic culture of an adult brain tissue sample, said nutrient medium comprising (i) one or more pH buffering system(s); (ii) one or more inorganic salt(s); (iii) one or more trace element(s); (iv) one or more free amino acid(s); (v) one or more vitamin(s); (vi) one or more hormone(s); (vii) one or more carbon/energy source(s); wherein said nutrient medium presents an osmolarity of from about 290 mOsm to about 330 mOsm, preferably of from about 300 mOsm to about 320 mOsm, most preferably of about 310 mOsm; wherein said nutrient medium has a pH of from about 7.0 to about 7.4, preferably of from about 7.1 to about 7.3, most preferably of about 7.2; and wherein said nutrient medium presents a glutamine content and a glutamic acid content of

from about 1 to about 20 μM , preferably of from about 5 μM to about 10 μM , most preferably of from about 7 μM to about 9 μM .

A second aspect of the present invention relates to a use of a nutrient medium for the in vitro organotypic culture of an adult brain tissue sample.

A third aspect of the present invention relates to a method for in vitro maintaining a neuronal and a glial cellular activity of an adult brain tissue sample comprising a step of incubating said adult brain tissue sample in said nutrient medium in conditions allowing survival of neuronal and glial cells.

LEGENDS OF THE FIGURES

Figure 1: Comparison of activity generated by slices of an adult brain tissue sample maintained in culture in MEM or a defined culture medium according to the invention ('OSCM'). Spontaneous activity recorded on MEM based medium (A) or 'OSCM' (B) at 9 days of culture. Interictal like activity (o) and simple or multi-unit activity (•) are only present when the adult brain tissue sample was in culture in 'OSCM'. Probability of recording multi-unit (C) or epileptiform (D) activities is higher for organotypic slices cultured with 'OSCM' ('defined medium', gray bars) as compared to the MEM based medium ('MEM', white bars).

Figure 2: Electrical activity over the time in culture with a culture medium according to the invention ('OSCM'). Spontaneous activity of slices of an adult brain tissue sample recorded on CA3 region at different time in culture using an 'OSCM' culture medium: 2 days (A), 10 days (B), 16 days (C) and 22 days (D).

Figure 3: Electrical activity over time in culture with a culture medium according to the invention ('OSCM'). (A) Graph representing a mean frequency of interictal like activity with respect to the time during which the slices of an adult brain tissue sample was kept in culture, either spontaneous (ACSF) or induced by a "convulsant solution" comprising high potassium (8 mM) and low magnesium (0.25 mM). (B) Graph representing the difference between global activities on ACSF versus convulsant solution at different time in culture.

Figure 4: Synchronous and variation of electrical activities with time in culture in a culture medium according to the invention ('OSCM'). (A) represents spreading of epileptic activities from a focal to adjacent areas: Dentate Gyrus (DG), region CA2, region

CA3 and Subiculum (Sub). There are no synchronous activities of either the DG, CA2 or CA3 regions with Subicular epileptic activities. With prolonged convulsant condition, ictal like activities could be generated in dentate gyrus (B) as in subiculum.

Figure 5: Pharmacological modulation of CA3 activities at 10 days of culture.

5 Interictal like activities appears spontaneously on CA3 region of the hippocampus (A). The event frequency is increase in convulsant condition (B): 8mM K⁺ and 0,25mM Mg²⁺. During wash out (C), initial activity is restored before being inhibited by GABAergic antagonist (picrotoxine, 50μM) application (D). Activities reappear during wash out (E) and are inhibited by Glutamatergic antagonist (10μM NBQX +100μM DL-APV) (F).

10 **Figure 6:** Pharmacological modulation of Cortical activities in organotypic culture. Temporal cortex slices are silent on MEM based medium (A) and in “OSCM”, either at d9 (B) or d24 (H), even if some isolated spikes could be recorded. After 9 days in culture, interictal like activity is revealed by “convulsant” conditions (B) that disappeared during wash out (D, F). At the opposite of the epileptic hippocampus, GABAergic
15 antagoniste (picrotoxine, 50μM) increase interictal activity (E) on the same manner than the potassium channel blocker (4AP, 50μM) (G).

Figure 7: Whole cell record from a subicular pyramidal cell in an organotypic slice. A current pulse of amplitude 400 pA and duration 2 sec is applied from the neuronal resting potential of -55 mV (A). Detail (B) of the signal from the rectangle in (A). A
20 hyperpolarizing current injection of amplitude 400 pA and duration 200 msec applied from -55 mV is applied (C).

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a novel nutrient medium that is notably adapted
25 for the *in vivo* culture of brain-derived cells, the said nutrient medium being especially useful for the *in vitro* organotypic culture of brain-derived tissue samples.

This invention pertains to a nutrient medium of defined composition for the *in vitro* organotypic culture of an adult brain tissue sample, said nutrient medium comprising
30 (i) one or more pH buffering system(s); (ii) one or more inorganic salt(s); (iii) one or more trace element(s); (iv) one or more free amino acid(s); (v) one or more vitamin(s); (vi) one or more hormone(s); (vii) one or more carbon/energy source(s); wherein said nutrient medium presents an osmolarity of from about 290 mOsm to about 330 mOsm, preferably

of from about 300 mOsm to about 320 mOsm, most preferably of about 310 mOsm; wherein said nutrient medium has a pH of from about 7.0 to about 7.4, preferably of from about 7.1 to about 7.3, most preferably of about 7.2; and wherein said nutrient medium presents a glutamine content and a glutamic acid content of from about 1 to about 250 μ M, preferably of from about 1 μ M to about 150 μ M, most preferably of from about 5 μ M to about 20 μ M.

It is shown in the examples herein that that temporal lobe tissue from adult epileptic patients can be maintained for up to 4 weeks in organotypic culture when the said tissue sample is incubated in the nutrient medium as described herein. It is also shown that epileptiform activities generated by these slices in culture in a nutrient medium as described herein are similar to those of acutely prepared slices from human, epileptic temporal lobe. The nutrient medium provided by the present invention permits long-term studies including the transfection of novel proteins and the identification of newly generated cellular elements.

It is also shown herein that in in vitro culture of brain-derived tissue samples in a nutrient medium as described herein, neurons survive and epileptiform activity is maintained for at least 3-4 weeks. Spontaneous activity was initiated in the subiculum and in the CA2/CA3 region as in acute slices of human epileptic tissue and possessed a similar sensitivity to receptor antagonists to acutely prepared slices.

The above-described advantageous properties of the nutrient medium that is described herein are useful, notably, for the purpose of assaying in vitro models mimicking animal models and patient trials for tests on novel anti-epileptic molecules.

Values for concentrations, pH units, weights and weight ratios are intended to further define the invention. Hence, it has to be understood that the term “about” refers to a range of values that comprise the explicitly given value and further extends to 10% below or above this given value.

The expression “defined composition” is intended to relate to a composition in which all added compounds have a known and traceable origin, and does not require any compound that originates from an animal source. Example of such a compound that originates from an animal source may be a compound that is found in animal serum, for example foetal calf serum, bovine serum or horse serum.

The terms “*nutrient medium*”, “*nutrient media*”, “*nutrient medium composition*” and “*nutrient medium compositions*” may be used interchangeably herein.

The expression “one or more” is intended to mean “at least one”, “at least two”, “at least three”, “at least four”, “at least five”, “at least six”, “at least seven”, “at least eight”, “at least nine”, “at least ten”, “at least eleven”, “at least twelve”, “at least thirteen”, “at least fourteen”, “at least fifteen”, “at least sixteen”, “at least seventeen”, “at least eighteen”, “at least nineteen”, “at least twenty”, “at least twenty one”, “at least twenty two”, and encompasses one, two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, fifteen, sixteen, seventeen, eighteen, nineteen, twenty, twenty one and twenty two, whenever applicable.

1) Nutrient medium

Important features of the inventive nutrient medium described herein encompass a particular range of osmolarities, a specific amount of free amino acids and vitamins as compared to the prior art, a particular range of pH, which aspects include the absence of HEPES in the inventive nutrient medium. Osmolarity of the nutrient medium according to the present invention ranges from about 290 mOsm to about 330 mOsm (milliosmols per liter of solution), whereas the pH of the nutrient medium ranges from about 7.0 to about 7.4, and most preferably ranges from 7.1 to 7.3.

The inventors have found that the features disclosed below are the basis for the nutrient medium hereinafter designated “OSCM”, for (Organotypic Slice Culture Medium).

A nutrient medium suitable for the present invention is preferably an aqueous liquid medium that maintain at least a basal metabolism allowing cells, especially cells contained in a brain-derived tissue sample, to survive.

(i) pH buffering system(s)

The nutrient medium according to the present invention comprises one or more pH buffering system(s).

A pH buffering system that is present in the nutrient medium according to the instant invention enables to maintain a pH of from about 7.0 to about 7.4, preferably of from about 7.1 to about 7.3, most preferably of about 7.2, during the course of the *in vitro* culture, e.g. in *in vitro* culture conditions in a 5% v/v CO₂ atmosphere in air.

In preferred embodiments, a pH buffering system that is present in the nutrient medium according to the instant invention enables to maintain a pH of from 7.0 to 7.4, preferably of from 7.1 to 7.3, most preferably of 7.2, during the course of the *in vitro* culture.

5 In some embodiments, the pH buffering system comprises a carbonic acid (H_2CO_3) and bicarbonate (HCO_3^-) pH buffering system.

In some embodiments, the pH buffering system comprises a phosphate-based pH buffering buffer.

10 In some embodiments, the phosphate-based pH buffering system is selected in a group comprising a phosphoric acid (H_3PO_4) and dihydrogen phosphate (H_2PO_4^-) pH buffering system, a potassium dihydrogen phosphate (KH_2PO_4) and di-potassium hydrogen phosphate (K_2HPO_4) pH buffering system, a sodium dihydrogen phosphate (NaH_2PO_4) and di-sodium hydrogen phosphate (Na_2HPO_4) pH buffering system, a sodium dihydrogen phosphate (NaH_2PO_4) and di-potassium hydrogen phosphate (K_2HPO_4) pH
15 buffering system and a potassium dihydrogen phosphate (KH_2PO_4) and di-sodium hydrogen phosphate (Na_2HPO_4) pH buffering system.

In some preferred embodiments, the pH buffering system comprises (i) a carbonic acid (H_2CO_3) and bicarbonate (HCO_3^-) pH buffering system and (ii) a phosphate-based pH buffering system.

20 In some other preferred embodiments, the pH buffering system comprises (i) a carbonic acid (H_2CO_3) and bicarbonate (HCO_3^-) pH buffering system and (ii) a phosphoric acid (H_3PO_4) and dihydrogen phosphate (H_2PO_4^-) pH buffering system.

25 Although HEPES is usually comprised in commercially available nutrient media, it is also known to potentially interfere with the electrophysiological activities of the cells contained in a brain tissue sample (see Bonnet *et al.* 1998).

Thus, in most preferred embodiments of the nutrient medium composition according to the present invention, the pH buffering system is HEPES-free, *i.e.* does not contain HEPES.

30 (ii) inorganic salts

The nutrient medium according to the present invention comprises one or more inorganic compound(s) that is/are selected in a group comprising calcium, chloride,

magnesium, phosphorus, potassium, sodium, sulphur. These inorganic compound(s) are most preferably present in the nutrient medium under the form of salts.

The one or more inorganic salt(s) that are contained in the nutrient medium according to the present invention is/are preferably selected in a group comprising calcium bromide, calcium chloride, calcium phosphate, calcium nitrate, calcium nitrite, calcium sulphate, magnesium bromide, magnesium chloride, magnesium sulphate, potassium bicarbonate, potassium bromide, potassium chloride, potassium dihydrogen phosphate, potassium disulphate, di- potassium hydrogen phosphate, potassium nitrate, potassium nitrite, potassium sulphite, potassium sulphate, sodium bicarbonate, sodium bromide, sodium chloride, sodium disulphate, sodium hydrogen carbonate, sodium dihydrogen phosphate, di-sodium hydrogen phosphate, sodium sulphate and a mix thereof.

In preferred embodiments, calcium, magnesium, potassium, phosphorus and sulphur may each be present in the nutrient medium at a concentration ranging from about 0.01 mM to about 100 mM, advantageously at a concentration ranging from about 0.1 mM to about 10 mM, and preferably at a concentration ranging from about 0.5 mM to about 5 mM.

In preferred embodiments, chloride and sodium may each be present in the nutrient medium at a concentration ranging from about 1 to about 1,000 mM, advantageously at a concentration ranging from about 50 mM to about 250 mM, and preferably at a concentration ranging from about 100 mM to about 150 mM.

(iii) trace elements

A “*trace element*” encompassed by the instant invention may also be referred to as a “*micronutrient*”. Within the scope of this invention both terms are considered to be equivalent and may substitute one another. Trace elements are required for the mitochondrial metabolism of both the neurons and the glial cells that are found in an adult brain tissue sample.

In some embodiments, the nutrient medium according to the present invention comprises one or more trace element(s) that is/are selected in a group comprising copper (Cu), iron (Fe), manganese (Mn), selenium (Se) and zinc (Zn).

In some preferred embodiments, the nutrient medium according to the invention comprises manganese. In these embodiments, manganese is preferably present in

the nutrient medium at a concentration ranging from about 0.01 nM to about 10 nM, preferably at a concentration ranging from about 0.05 nM to about 5 nM.

In some preferred embodiments, manganese is present under the form of a manganese salt, e.g. under the form of manganese chloride ($\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$).

5 In some preferred embodiments, copper may be present in the nutrient medium at a concentration ranging from about 0.1 nM to about 100 nM, advantageously at a concentration ranging from about 1 nM to about 25 nM, preferably at a concentration ranging from about 5 nM to about 15 nM.

10 In a still preferred embodiment, copper is present under the form of a copper salt, e.g. copper sulphate ($\text{Cu(II)SO}_4 \cdot 5\text{H}_2\text{O}$).

In some preferred embodiments, selenium is present in the nutrient medium at a concentration ranging from about 0.1 nM to about 100 nM, advantageously at a concentration ranging from about 1 nM to about 50 nM, preferably from 20 nM to about 40 nM in the nutrient medium according to the present invention.

15 In one embodiment, iron and zinc may each represent from about 0.10 μM to about 10 μM , preferably from about 0.25 μM to about 2 μM , preferably from about 0.6 μM to about 1.5 μM in the nutrient medium according to the present invention.

In a still preferred embodiment, iron may originate from ferric(III) nitrate ($\text{Fe(III)NO}_3 \cdot 9\text{H}_2\text{O}$) and/or ferric(II) sulphate ($\text{Fe(II)SO}_4 \cdot 7\text{H}_2\text{O}$).

20

(iv) free amino acids

As used herein a “*free amino acid*” is an amino acid molecule that is not included in a peptide or in a protein. Free amino acids are compounds that are contained in the constitution of most of culture medium compositions.

25 The nutrient medium according to the present invention comprises one or more free amino acid(s) that is/are selected in a group comprising L-alanine; L-arginine; L-asparagine; L-aspartic acid; L-cysteine; L-cystine; L-glutamine; L-glutamic acid; glycine; L-histidine; L-isoleucine; L-leucine; L-lysine; L-methionine; L-phenylalanine; L-proline; L-serine; taurine; L-threonine; L-tryptophan; L-tyrosine; L-valine.

30 Any combination of the 20 “*conventional*” free amino acids is encompassed by the instant invention : L-alanine; L-arginine; L-asparagine; L-aspartic acid; L-cysteine; L-glutamine; L-glutamic acid; glycine; L-histidine; L-isoleucine; L-leucine; L-lysine; L-

methionine; L-phenylalanine; L-proline; L-serine; L-threonine; L-tryptophan; L-tyrosine; L-valine.

In some preferred embodiments, the nutrient medium according to the instant invention comprises a combination of two or more free amino acids which are selected in a group comprising the 20 “*conventional*” free amino acids described above.

As intended herein, the nutrient medium according to the invention comprises 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 distinct “*conventional*” free amino acids.

In some embodiments, the nutrient medium according to the present invention may also comprise one or more “*non-conventional*” free amino acids, such as L-cystine and taurine.

In some embodiments, one or more free amino acid(s) that is/are present in the nutrient medium according to the instant invention, with the exception of glutamine and glutamic acid, is/are comprised at a concentration ranging from about 0.1 μM to about 2,000 μM , preferably at a concentration ranging from about 1 μM to about 1,000 μM , most preferably at a concentration ranging from about 5 μM to about 250 μM .

In some embodiments, each of the amino acids selected in the group comprising L-alanine, L-asparagine, L-aspartic acid, taurine and L-tryptophane, when present in the nutrient medium according to the invention, is preferably contained in the said nutrient medium at a concentration ranging from about 1 μM to about 20 μM , preferably from about 5 μM to about 10 μM .

In preferred embodiments, the nutrient medium of the invention contains taurine. The presence of taurine is of special interest for the maintenance of glial cells metabolism.

In some embodiments, L-cysteine, when present in the nutrient medium of the invention, is advantageously contained in the said nutrient medium at a concentration ranging from about 0.1 μM to about 500 μM , preferably at a concentration ranging from about 1 μM to about 100 μM , most preferably at a concentration ranging from about 1 μM to about 20 μM .

In some embodiments, each of the amino acids selected in the group comprising L-cystine, L-histidine, L-methionine, L-proline, when present in the nutrient medium of the invention, is advantageously contained in the said nutrient medium at a

concentration ranging from about 0.1 μM to about 600 μM , preferably at a concentration ranging from about 1 μM to about 150 μM , most preferably at a concentration ranging from about 5 μM to about 50 μM .

In some embodiments, each of the amino acids selected in the group comprising glycine, L-phenylalanine, L-serine, L-tyrosine, when present in the nutrient medium of the invention, is advantageously contained in the said nutrient medium at a concentration ranging from about 0.1 μM to about 1,000 μM , preferably at a concentration ranging from about 1 μM to about 250 μM , most preferably at a concentration ranging from about 5 μM to about 100 μM .

In some embodiments, each of the amino acids selected in the group comprising L-arginine, L-isoleucine, L-lysine, L-leucine, L-threonine, L-valine, when present in the nutrient medium of the invention, is advantageously contained at a concentration ranging from about 0.1 μM to about 2,000 μM , preferably at a concentration ranging from about 1 μM to about 500 μM , most preferably at a concentration ranging from about 50 μM to about 150 μM .

In preferred embodiments, the nutrient medium according to the present invention has a content in the combination of glutamine and glutamic acid free amino acids each ranging from about 1 μM to about 250 μM , preferably ranging from about 1 μM to about 150 μM , most preferably ranging from about 5 μM to about 20 μM .

Glutamine and glutamic acid are usually present in the millimolar (mM) range in nutrient medium (Jung *et al.* (2002); Chaichana *et al.* (2007); US Patent n° 4,657,866).

Others also reported nutrient mediums for culturing neural cells having an amount of glutamine, in the millimolar range, whereas the glutamic acid content ranges in the nanomolar range (US 2012/0052577; US Patent n° 5,910,443).

At physiological pH, i.e. at a pH ranging from 7.35 to 7.45, glutamic acid is negatively charged, hence under its glutamate form. It is known in the art that glutamate is a neurotransmitter and a precursor of GABA. It has been previously found that glutamate may be toxic at a concentration above 100 μM . Hence, it is preferable of keeping the glutamate content in the nutrient medium as defined herein as low as possible to avoid its toxicity. Moreover, glutamine is metabolized by glial cells into glutamate, in turn feeding the neurons with this neurotransmitter. Hence, in order to avoid large amount of glutamate neurotransmitter to be present in the nutrient medium according to the instant invention,

the glutamate and the glutamine content is believed to be advantageously as low as possible, preferably below 250 μM .

(v) vitamins

5 The nutrient medium according to the present invention comprises one or more vitamin(s) that is/are selected in a group comprising biotin (vitamin H); D-calcium-pantothenate; choline chloride; folic acid (vitamin B9); myo-inositol; nicotinamide; pyridoxal (vitamin B6); riboflavin (vitamin B2); thiamine (vitamin B1); cobalamin (vitamin B12); acid ascorbic; α -tocopherol (vitamin E) and a combination of two or more
10 vitamins thereof.

 In some embodiments, cobalamin (vitamin B12) may be contained in the nutrient medium of the invention at a concentration ranging from about 0.001 μM to about 1,500 μM , preferably at a concentration ranging from about 0.01 μM to about 0.15 μM , most preferably at a concentration ranging from about 0.01 μM to about 0.10 μM .

15 In some embodiments, riboflavin (vitamin B2) may be contained in the nutrient medium of the invention at a concentration ranging from about 0.001 μM to about 2,500 μM , preferably at a concentration ranging from about 0.01 μM to about 0.500 μM , most preferably at a concentration ranging from about 0.08 μM to about 0.15 μM .

 In some embodiments, biotin (vitamin H) may be contained in the nutrient
20 medium of the invention at a concentration ranging from about 0.01 μM to about 10 μM , preferably at a concentration ranging from about 0.1 μM to about 5 μM , most preferably from about 0.4 μM to about 0.8 μM .

 In some embodiments, each of the vitamins selected in the group comprising D-calcium pantothenate, folic acid (vitamin B9) and thiamine (vitamin B1), when present,
25 may be contained in the nutrient medium of the invention at a concentration ranging from about 0.01 μM to about 10 μM , preferably at a concentration ranging from about 0.1 μM to about 5.0 μM , most preferably at a concentration ranging from about 1 μM to about 1.5 μM .

 In some embodiments, α -tocopherol, when present, may be contained in the
30 nutrient medium of the invention at a concentration ranging from about 0.01 μM to about 100 μM , preferably at a concentration ranging from about 0.1 μM to about 25 μM , most preferably at a concentration ranging from about 2 μM to about 6 μM .

In some embodiments, α -tocopherol, when present, may be provided in the nutrient medium according to the present invention as a mixture of α -tocopherol and α -tocopherol acetate.

In some preferred embodiments of the nutrient medium according to the present invention wherein α -tocopherol is present, the said nutrient medium may contain equimolar amounts of α -tocopherol and α -tocopherol acetate.

In some embodiments, myo-inositol, when present, may be contained at a concentration ranging from about 0.01 μ M to about 250 μ M, preferably at a concentration ranging from about 0.5 μ M to about 100 μ M, most preferably at a concentration ranging from about 10 to about 15 μ M.

In some embodiments, each of the vitamins selected in the group comprising choline chloride, nicotinamide and pyridoxal (vitamin B6), when present, may be contained in the nutrient medium of the invention at a concentration ranging from about 0.01 μ M to about 100 μ M, preferably at a concentration ranging from about 0.1 μ M to about 50 μ M, most preferably at a concentration ranging from about 1 μ M to about 15 μ M.

In one embodiment, ascorbic acid, when present, may be contained in the nutrient medium of the invention at a concentration ranging from about 10 μ M to about 5,000 μ M, preferably at a concentration ranging from about 50 μ M to about 2,500 μ M, most preferably at a concentration ranging from about 250 μ M to about 1,000 μ M.

(vi) hormones

The Nutrient medium according to the invention comprises one or more hormone(s) that is/are selected in a group comprising insulin; 17 β -estradiol; human transferrin; progesterone; corticosterone; triiodothyronine (T3) and a mix thereof.

In some embodiments, 17 β -estradiol, when present, may be contained in the nutrient medium of the invention at a concentration ranging from about 0.01 nM to about 20 nM, preferably at a concentration ranging from about 0.1 nM to about 5 nM, most preferably at a concentration ranging from about 0.75 nM to about 1.50 nM.

In some embodiments, triiodothyronine (T3), when present, may be contained in the nutrient medium of the invention at a concentration ranging from about 0.1 nM to about 100 nM, preferably at a concentration ranging from about 1 nM to about 50 nM, most preferably at a concentration ranging from about 2 nM to about 5 nM.

In some embodiments, progesterone, when present, may be contained in the nutrient medium of the invention at a concentration ranging from about 0.1 nM to about 250 nM, preferably at a concentration ranging from about 1nM to about 100 nM, most preferably at a concentration ranging from about 5 nM to about 25 nM.

5 In some embodiments, corticosterone, when present, may be contained in the nutrient medium of the invention at a concentration ranging from about 0.1 nM to about 1,000 nM, preferably at a concentration ranging from about 1 nM to about 250 nM, most preferably at a concentration ranging from about 10 nM to about 100 nM.

10 In some embodiments, insulin, when present, may be contained in the nutrient medium of the invention at a concentration ranging from about 0.1 nM to about 100 μ M, preferably at a concentration ranging from about 1 nM to about 10 μ M, most preferably at a concentration ranging from about 100 nM to about 1,000 nM.

15 In some embodiments, human transferrin, when present, may be contained in the nutrient medium of the invention at a concentration ranging from about 0.1 μ M to about 100 μ M, preferably at a concentration ranging from about 1 μ M to about 25 μ M, most preferably at a concentration ranging from about 1 μ M to about 10 μ M.

In a preferred embodiment, the human transferrin is provided in the form of a recombinant hormone.

20 (vii) carbon/energy sources

The Nutrient medium according to the invention comprises one or more carbon/energy source(s) which is/are preferably selected in a group comprising D-glucose; pyruvate; lactate; ATP; creatine; creatine phosphate; and a mix thereof.

25 It is needless to mention that D-glucose, pyruvate, lactate and ATP, from the nutrient medium according to the instant invention, when present, may be considered as direct or indirect available energy sources for the metabolism of neurons and glial cells that are present in the adult brain tissue sample.

30 In some further embodiments, the nutrient medium of the invention may contain one or more compounds selected in the group comprising creatine and creatine phosphate.

Creatine and creatine phosphate are intended to contribute to the regeneration of ATP from ATP breakdown products, such as ADP and AMP.

In one embodiment, ATP may represent from about 0.01 μM to about 1,000 μM , preferably from about 0.1 μM to about 500 μM , preferably from about 1 μM to about 200 μM in the nutrient medium according to the present invention.

5 In one embodiment, creatine phosphate and pyruvate may each represent from about 1 μM to about 10,000 μM , preferably from about 10 μM to about 1,000 μM , preferably from about 100 μM to about 500 μM in the nutrient medium according to the present invention.

10 In one embodiment, creatine, glucose and lactate may each represent from about 0.01 mM to about 100 mM, preferably from about 0.05 mM to about 50 mM, preferably from about 0.1 mM to about 20 mM in the nutrient medium according to the present invention.

In one embodiment, the nutrient medium according to the present invention may comprise at least two carbon/energy sources, said carbon/energy sources comprising lactate and pyruvate.

15 In a preferred embodiment, lactate and pyruvate are preferably present in a weight ratio lactate/pyruvate of from about 1 to about 100, preferably from about 5 to about 50, preferably from about 15 to about 25, preferably of from about 17 to about 21, most preferably of about 19.

20 (viii) Osmolarity

The nutrient medium according to the present invention presents an osmolarity of from about 290 to about 330 mOsm, preferably of from about 300 to about 320 mOsm, most preferably of about 310 mOsm.

25 Osmolarity may be determined according to any one of the methods that are well known from the one skilled in the art. Osmolarity is most preferably measured using a vapour osmometer 5520 from Wescor company (<http://www.wescor.com>).

In one embodiment, the osmolarity of the nutrient medium is measured after equilibration with 5% CO_2 at room temperature.

30 Osmolarity depends on the content of the various inorganic compounds and their ratios, the content of the pH buffering system(s) used therein, the balance between acid and basic compounds in the nutrient medium.

Inventors surprisingly show that the physiological range of osmolarity, in combination with the other features tends to provide a suitable nutrient medium of *in vitro* organotypic cultures of adult brain tissue sample. Indeed, the envisioned osmolarity allows the neurons and the glial cells to maintain a metabolism and/or an electrical activity that mimic the conditions observed *in vivo*.

(ix) pH

The nutrient medium according to the present invention has a pH ranging from about 7.0 to about 7.4, preferably ranging from about 7.1 to about 7.3, and is most preferably of about 7.2.

In one embodiment, the pH of the nutrient medium is measured after equilibration with 5% CO₂ at 37°C.

The inventors believe that the pH of the nutrient medium according to the instant invention is compatible with a physiological pH, i.e. a pH value that the neurons and the glial cells experience *in vivo*.

Hence the pH of the nutrient medium according to the instant invention allows for a metabolism and electrical activities that mimic the conditions observed *in vivo*.

(x) additional compounds

The Nutrient medium according to the invention comprises one or more additional compound(s) which is/are preferably selected in a group comprising oxaloacetic acid; glutathione; glycerol; acetic acid; citric acid; thymidine; lipoic acid; linoleic acid; hypoxanthine; β -hydroxybutyrate and a mix thereof.

In some further embodiments, the nutrient medium of the invention may contain intermediate metabolites of the tricarboxylic acid cycle, preferably selected in a group comprising acetate, citrate, oxaloacetate and a mix thereof.

In a still preferred embodiment, the intermediate metabolites of the tricarboxylic acid cycle are brought preferably in the form of an acid, preferably selected in a group comprising acetic acid, citric acid, oxaloacetic acid and a mix thereof.

In some further embodiments, the nutrient medium of the invention may contain thymidine. In these further embodiments, thymidine may be contained in the nutrient medium of the invention at a concentration ranging from about 0.01nM to about

5,000 nM, preferably at a concentration ranging from about 0.1 nM to about 500 nM, most preferably at a concentration ranging from about 1 nM to about 250 nM.

Lipoic acid; linoleic acid; hypoxanthine; β -hydroxybutyrate may each contribute to the lipid metabolism of the cells cultured in the nutrient medium of the invention, especially to the lipid metabolism of neurons and glial cells that are present in the adult brain tissue sample.

Linoleic acid, when present, may be contained in the nutrient medium of the invention at a concentration ranging from about 0.01 nM to about 500 nM, preferably at a concentration ranging from about 0.1 nM to about 100 nM, most preferably at a concentration ranging from about 1 nM to about 50 nM.

In some embodiments, lipoic acid, when present, may be contained in the nutrient medium of the invention at a concentration ranging from about 0.01 nM to about 1,000 nM, preferably at a concentration ranging from about 0.1 nM to about 250 nM, most preferably at a concentration ranging from about 1 nM to about 100 nM.

In the embodiments of the nutrient medium wherein hypoxanthine is present, hypoxanthine may be contained therein at a concentration ranging from about 0.01 μ M to about 1,000 μ M, preferably from about 0.1 μ M to about 250 μ M, most preferably from about 0.5 μ M to about 10 μ M.

In the embodiments of the nutrient medium wherein glutathione is present, may be contained therein at a concentration ranging from about 0.01 μ M to about 1,000 μ M, preferably from about 0.1 μ M to about 250 μ M, most preferably from about 0.5 μ M to about 10 μ M.

In one embodiment, glycerol may represent from about 0.01 μ M to about 1,000 μ M, preferably from about 0.1 μ M to about 500 μ M, preferably from about 1 μ M to about 200 μ M in the nutrient medium according to the present invention.

In one embodiment, citric acid may represent from about 0.001 nM to about 100 mM, preferably from about 0.01 nM to about 25 mM, preferably from about 0.1 nM to about 1 mM in the nutrient medium according to the present invention.

In one embodiment, acetic acid, β -hydroxybutyrate and oxaloacetic acid may each represent from about 0.01 mM to about 100 mM, preferably from about 0.05 mM to about 50 mM, preferably from about 0.1 mM to about 20 mM in the nutrient medium according to the present invention.

In some embodiments, the nutrient medium according to the present invention may also comprise BSA fraction V. BSA fraction V may be used for some compounds to enter the cells and is of special interest to solubilize the lipophilic compounds within the nutrient medium according to the instant invention.

5 In some embodiments, BSA fraction V is present in an amount from about 0.01 to about 1,000 μM , preferably from about 0.1 μM to about 200 μM , most preferably from about 1 μM to about 100 μM .

 In some embodiments, the nutrient medium according to the present invention may also comprise one or more antioxidant compound(s), preferably selected in a group
10 comprising putrescine; superoxide dismutase (SOD); ascorbic acid; citric acid; acetic acid; glutathione; selenium and mixtures of two or more compounds thereof.

 In some embodiments, the one or more antioxidant(s) is/are present in an amount from about 0.0001 μM to about 10,000 μM , preferably from about 0.001 μM to about 5,000 μM , most preferably from about 0.01 μM to about 2,000 μM .

15 In some embodiments, putrescine, when present, may be contained in the nutrient medium of the invention at a concentration ranging from about 0.01 μM to about 1,000 μM , preferably at a concentration ranging from about 0.1 μM to about 250 μM , most preferably at a concentration ranging from about 1 μM to about 100 μM .

 In some embodiments, the nutrient medium according to the present invention
20 may further comprise one or more neurotransmitter(s) and/or one or more precursor(s) thereof, preferably selected in a group comprising GABA, glutamate; glycine; aspartate; histidine; tryptophane; tyrosine; choline; phenylalanine and a mix thereof.

 In some embodiments, the one or more neurotransmitter(s) and/or the one or more precursor(s) thereof is/are present in an amount from about 0.001 μM to about
25 10,000 μM , preferably from about 0.01 μM to about 1,000 μM , most preferably from about 0.1 μM to about 500 μM .

 In some embodiments, the nutrient medium according to the present invention may further comprise one or more antibiotic(s), preferably selected in a group comprising amphotericin B, penicillin, streptomycin and a mix thereof.

30

(xi) adult brain tissue sample

The expression “*adult brain tissue sample*” encompasses brain tissue sample that originates from a fully developed individual, wherein growth and sexual maturity are achieved. Hence, the expression “*adult brain tissue sample*” does not encompass
 5 “*embryonic*”, “*foetal*”, “*prenatal*”, “*perinatal*”, “*postnatal*” and “*natal*” brain tissue sample.

Within the scope of the present invention, an adult brain tissue sample encompassed herein may comprise a tissue sample from an adult cerebral cortex selected in a group comprising a tissue sample of a frontal lobe, a tissue sample of a parietal lobe, a
 10 tissue sample of an occipital lobe, a tissue sample of a temporal lobe, a tissue sample of limbic lobe and a tissue sample of the cerebellum.

In some embodiments, the adult brain tissue sample comprises a tissue sample of frontal lobe comprising the precentral gyrus, the superior frontal gyrus, the middle frontal gyrus, the inferior frontal gyrus, the pars opercularis, the pars triangularis, the pars
 15 orbitalis, the medial frontal gyrus, the paracentral lobule, the rectus gyrus, the orbital gyrus, the precentral gyrus,, the rolandique lobule and a mix thereof.

In some embodiments, the adult brain tissue sample comprises a sample of parietal lobe comprising the poscentral gyrus, the superior parietal lobule, the inferior parietal lobule, the supramarginal gyrus, paracentral lobule, the angularis gyrus, the
 20 precuneus and a mix thereof.

In some embodiments, the adult brain tissue sample comprises a sample of occipital lobe comprising the lateral occipital gyrus, the superior occipital gyrus, the inferior occipital gyrus, the cuneus, the lingual gyrus, the occipitotemporal gyrus, the calcarine sulcus, the internal occipital pole and a mix thereof.

In some embodiments, the adult brain tissue sample comprises a sample of temporal lobe comprising the superior temporal gyrus, the middle temporal gyrus, the inferior temporal gyrus, the lingual gyrus, the occipitotemporal gyrus, the medial occipitotemporal gyrus, the lateral occipitotemporal gyrus the enthorinal cortex and the perirhinal cortex and a mix thereof.

In some embodiments, the adult brain tissue sample comprises a sample of limbic lobe comprising the cingulate gyrus, the isthmus of cingulate gyrus, the parahippocampal gyrus, the uncus, the uncinate gyrus, the hippocampal formation, the

hippocampus, the Ammon's horn, the dentate gyrus, the fasciolar gyrus, the indusium griseum, the paraterminal gyrus, the subcallosal area and a mix thereof.

In some embodiments, an adult brain tissue sample comprises a sample of hippocampus and a sample of temporal lobe.

5 In some embodiments, the adult brain tissue sample originates from a nonhuman or a human individual, preferably from a human individual.

In some embodiments, the nonhuman individual is a nonhuman mammal, preferably a primate or a rodent.

10 In some embodiments, the adult brain tissue sample originates from a biopsy or a therapeutic resection.

In some embodiments, the adult brain tissue sample according to the instant invention has a thickness from about 10 to about 2,000 μm , preferably from about 100 to about 1,000 μm , most preferably from about 200 to about 500 μm .

15 2) Preparation of a nutrient medium according to the invention

A nutrient medium according to the present invention may be prepared from individual separate ingredients, commercially available as culture grade powders, solutions, suspensions or emulsions.

20 Alternatively, a basic medium may be achieved using commercially available minimum medium, provided said commercial medium is compatible with the glutamine and glutamate contents.

Suitable starting media that may be used for preparing a nutrient medium according to the present invention encompass the media selected in a group comprising BME, DMEM/F12, HBSS, MEM, and mixture of two or more media thereof.

25 The nutrient medium according to the present invention may be prepared by mixing two or more stock solutions.

In a preferred embodiment, the nutrient medium according to the invention is an aqueous liquid solution.

30 In another preferred embodiment, the nutrient medium according to the invention is sterilized before use. Sterilization may be achieved by any suitable method known from a skilled in the art.

The present invention also relates to a composition for preparing a nutrient medium as described in the present specification.

In some embodiments, the said composition is under the form of a powder composition comprising the compounds required for preparing a given volume of the nutrient medium that is described in the present specification.

Thus, this invention also relates to a powder composition comprising a combination of compounds for use for preparing a liquid nutrient medium as described in the present specification.

In preferred embodiments, the said liquid nutrient medium may be prepared by adding the desired amount of water to a determined amount of the powder composition. In preferred embodiments, the said water consists of sterile demineralized water.

In some embodiments, sterile demineralized water suitable for implementing the instant invention is endotoxin free water.

3) Uses of the nutrient medium

In some embodiments, the nutrient medium as described herein, may be used for the *in vitro* culture of an adult brain tissue sample, which encompasses an adult hippocampus and/or temporal cortex tissue sample.

In some embodiments, the nutrient medium according to the instant invention may be used for the *in vitro* organotypic culture of an adult brain tissue sample that originates from a human individual presenting a condition selected in a group comprising epilepsy, brain tumor and demyelinating neuropathology.

Among brain tumors, one may cite glioblastoma, anaplastic astrocytoma, oligodendroglioma, ependymoma, ganglioglioma, medulloblastoma, brain sarcoma, brain adenocarcinoma, chordoma, hemangioblastoma, meningioma, osteoma, pinealoma, pituitary adenoma and schwannoma, dysenbryoplastic neuroepithelial tumor (DNET).

In some embodiments, the present invention relates to the use of the nutrient medium according to the instant invention, wherein the adult brain tissue sample originates from a human individual presenting an epileptic condition.

Surgeons routinely use a physiological solution, typically 0.9% NaCl, for washing the surgical site. However, this physiological solution is not suitable when maintaining a neuronal activity is at stake.

In another embodiment, the nutrient medium according to the instant invention may be used for washing the surgical site.

In some embodiments, washing the surgical site is performed with a nutrient medium according to the instant invention that also comprises HEPES, preferably in an amount from about 1 mM to 100 mM, most preferably in an amount from about 10 mM to 50 mM.

In some embodiments, washing the surgical site is performed with a nutrient medium according to the instant invention.

10 4) Methods

The present invention relates to a method for *in vitro* maintaining a neuronal and a glial cellular activity of an adult brain tissue sample comprising a the step of incubating the said adult brain tissue sample in said nutrient medium in condition allowing survival of neuronal and glial cells.

15 The present invention also relates to a method for *in vitro* maintaining a neuronal and a glial cellular activity of an adult brain tissue sample comprising a the step of a) providing an adult brain tissue sample; b) contacting said adult brain tissue sample with a nutrient medium; c) incubating the said adult brain tissue sample in said nutrient medium according to the instant invention in conditions allowing survival of neuronal and
20 glial cells.

In a preferred embodiment, the instant invention relates to a method for *in vitro* maintaining a neuronal and a glial cellular activity of an adult brain tissue sample wherein said nutrient medium is changed every other day, preferably every day.

In one embodiment, condition allowing survival of neuronal and glial cells is
25 achieved by *in vitro* culturing an adult brain tissue sample with 5% CO₂ at 37°C.

5) Industrial application

The nutrient medium, the uses thereof and the methods encompassed by the instant application provide potential numerous applications, such as:

30 - rendering pharmacological assays for diagnostic or therapeutic purposes easier to achieve;

- testing for cell therapy: cell transplantation of a tissue in order to study the integration at the neuronal network and its repair;
- testing for gene therapy: transplantation of genetically modified or reprogrammed cells to express a therapeutic molecule, a cell effector. Safety test /
5 expression vectors neuronal viral tropism;
- providing basic research tools: a study of the electrophysiological signal processing and propagation within a structure, study of proliferation, migration, cell differentiation;
- providing an *in vitro* model for studying neurological autoimmune diseases
10 such as epilepsy, demyelinating neuropathologies, limbic encephalitis;
- providing an *in vitro* model for toxicological study: multiple chemical sensitivity syndrome;
- providing an *in vitro* model to make coronal studies between different regions: cornu ammoni, subiculum, entorhinal cortex, perirhinal cortex, temporal cortex
15 or other brain region subjected to a therapeutic resection;
- providing a real *ex vivo* model of human pathology without the need of extrapolate the results as the one obtained on animal model;
- providing a model *in vitro* to study the human neurogenesis as an ectopic once has been found on that kind of sample (Crespel *et al.* (2005)).

EXAMPLES

A. MATERIALS AND METHODS

A.1. Patients and tissue

95 slices for organotypic culture were prepared from 28 tissue blocks
25 (20 hippocampus, 8 temporal cortex) obtained from 21 patients (13 males and 8 females, from 20-55 years old) diagnosed with pharmaco-resistant mesial temporal lobe epilepsies usually associated with a hippocampal sclerosis. Patients gave a written, informed consent and our protocol was approved by the 'Comité Consultatif National d'Ethique'. Tissues were transported from the operating theatre to the laboratory in a cold, oxygenated sucrose
30 solution containing 250 mM sucrose; 25 mM NaHCO₃; 3 mM KCl; 1 mM CaCl₂; 10 mM MgCl₂; 10 mM glucose and equilibrated with 95% O₂ / 5% CO₂.

A.2. Preparation of organotypic cultures.

Capillaries and damaged tissue were removed from the tissue block in sterile conditions, in the same sucrose containing solution at 2-10 °C and equilibrated with 95% O₂ / 5% CO₂. Slices of thickness 300 µm were cut with a vibrating tissue slicer (HM650V, Microm). Slices of the hippocampal formation included the dentate gyrus, the often sclerotic CA3 region, the usually sclerotic CA1 region and the subiculum, and measured 5-6 mm by 10-15 mm. Slices of temporal cortex, obtained from the same operations and of size ~10-15 x 10-15 mm, were also prepared for organotypic culture. Some slices were fixed for subsequent anatomy. Slices used for culture were placed on an insert (30 mm Transwell Coll., Corning) at the interface between air and a culture medium according to the invention (namely 'OSCM'), further containing 20 mM HEPES and a mixture of antibiotics, penicillin (100 U/ml) and streptomycin (100 µg/ml). Slices were maintained in an incubator, at 37°C in 95% O₂ / 5% CO₂. After 1 hr, slices on culture inserts were transferred to a 6-well plate, pre-equilibrated in the incubator for 1 hr and containing the same culture medium (OSCM), except that HEPES was omitted. Culture medium (OSCM) was changed 5-6 times per week. After one week, the culture medium (OSCM) was replaced by a fresh culture medium, except it contains no antibiotics. Slices were then subjected to electrophysiological or morphological studies after one, two or three weeks in culture.

A.3. Organotypic culture medium.

Visual inspection and electrophysiological records suggested that brain slices from the temporal lobe of adult epilepsy patients did not survive well in cultures using either the culture medium used by Stoppini *et al.* (1991), a mixture of the minimum essential medium (MEM), horse serum and Hank's medium or the Neurobasal neuronal medium (Armentano *et al.* (2011)).

Table 1 gives the full formulation of the organotypic medium according to the instant invention, namely 'OSCM'. This medium does not include serum (Gähwiler (1988); Stoppini *et al.* (1991)). It relies on bicarbonate-based, rather than HEPES-based pH buffering system (Bonnet *et al.* (1998)). Inorganic ion concentrations are as follows: 3.3 mM K⁺; 145.5 mM Na⁺; 1.3 mM Ca⁺⁺; 1 mM Mg⁺⁺; 124 mM Cl⁻; 22.2 mM HCO₃⁻; 1.1 mM (PO₄); 0.9 mM SO₄⁻. Trace metals are present in the medium at the following

concentrations: 11 μM Cu^{+++} ; 699 μM Fe^{++} and Fe^{+++} ; 1 μM Mn^{++} and 715 μM Zn^{++} . The lactate/pyruvate ratio and adding oxaloacetate to the medium is believed to help neuronal recovery. It was also hypothesized that synchronous network activities that emerge from the rhythmic discharges, like gamma oscillations, consume a significant amount of energy.

- 5 The culture medium was therefore enriched with a mix of early and late energy sources and intermediate metabolites of the tricarboxylic acid cycle, to maintain epileptic activities over the time in culture: d-Glucose, pyruvate, lactate, acetate, citrate, oxaloacetate were used in complements with ATP, Creatine phosphate and Creatine. Several antioxidants compounds were also added: putrescine, SOD, ascorbic acid, citric acid, glutathione and
- 10 selenium. Amino acids content was lowered below concentrations found in many culture media, including MEM and Neurobasal (Brewer *et al.* (1993)). Several vitamins were present, also at lower concentrations than in many culture media. The pH of the medium was maintained with CO_2/HCO_3 and phosphate buffers, but HEPES was not used. The pH of this medium, equilibrated with 5% CO_2 at 37°C, was in the range 7.1 – 7.3 and
- 15 osmolarity was in the range 290-310 mOsm.

Table 1 : Composition of a defined culture medium “OSCM” suitable for organotypic cultures.

Type	Compounds	Concentration (μ M)
Inorganic salts	Calcium Chloride	1337.5
	Ferric(III) Nitrate	0.019
	Ferric (III) sulphate	0.68
	Potassium Chloride	3383.5
	Cupric(II) sulfate	0.01
	Magnesium sulphate	988
	Magnesium chloride	45.3
	Sodium Chloride	116878
	Sodium dihydrogen phosphate	1059.5
	di-Sodium hydrogen phosphate	75
	Sodium hydrogen Carbonate	22282
	Zinc sulphate	0.71
	Manganese chloride	0.001

Amino Acids	L-Glutamine	8
	L-Alanine	7.5
	L-Arginine	119.78
	L-Asparagine	7.5
	L-Aspartic acid	7.5
	L-Cysteine	14.97
	L-Glutamic acid	7.5
	L-Cystine	22.5
	Glycine	37.5
	L-Histidine	30.24
	L-Isoleucine	92.1
	L-Leucine	91.35
	L-Lysine	74.85
	L-Methionine	24.95
	L-Phenylalanine	47.25
	L-Proline	22.5
	Taurine	8
	L-Serine	37.5
	L-threonine	97.65
	L-tryptophan	9.57
	L-tyrosine	47.01
	L-valine	97.95

Vitamins	Biotin	0.61
	D-Calcium panthotenate	1.02
	Choline Choride	10.69
	Folic acid	1.24
	Myo-inositol	12
	Nicotinamide	3.72
	Pyridoxal	2.19
	Riboflavine	0.13
	Thiamine	1.41
	Cobalamine	0.08
	Ascorbic acid	580
	α -Tocopherol	2.3
	α -Tocopherol acetate	2.1
Hormones	Insulin	0.64
	T3	0.003
	17- β Oestradiol	0.0008
	Corticosterone	0.058
	Progesterone	0.01
Energetic sources	D-Glucose	4090
	Sodium Lactate	4000
	Creatine	5000
	Phosphate creatine	200
	Sodium Pyruvate	215
	ATP	10

Other	Sodium hypoxanthin	2.25
	Thymidine	0.22
	Lipoic acid	0.076
	Linoleic acid	0.022
	Putrescine	50.05
	Oxaloacetic acid	1000
	Heparine	50
	GABA	1
	Glycerol	50
	Citric acid	573
	Acetic acid	570
	Sodium β -Hydroxybutyrate	1000
	Mannitol	5000
	Transferrine	5
	BSA fraction V	40
	Glutathione reduced	3.25
	Selenium	0.015
	SOD	0.08

A.4. Multi-unit and field records from cultures in an interface chamber.

Survival of neurons and glial cells are assessed through the measurement of the electrical properties after several days in culture, which result from maintaining an active cellular metabolism. Extracellular electrical records were made from organotypic slices after 2-29 days in culture using 'OSCM'. Cultured slices were transferred on the culture insert to an interface recording chamber maintained at 35-7° C and equilibrated with 5% CO₂ in 95% O₂. Spontaneous activities were recorded on ACSF solution containing 124 mM NaCl, 26 mM NaHCO₃, 4 mM KCl, 2 mM MgCl₂, 2 mM CaCl₂, and 10 mM D-glucose. The same slice was then perfused with a 'convulsant solution', in which increased K⁺ to 8 mM and decreased Mg²⁺ to 0.25 mM enable to induce ictal-like activity. To modulated electrophysiological activities, records were made on ACSF supplemented with different effectors between a wash out period to verify that the original signal were restored: Ionotropic glutamate signalling was blocked with 2,3-dihydroxy-6-nitro-7-

sulfamoyl-benzo(f)quinoxaline (NBQX, 10 μ M) and DL-2-amino-5-phosphonovaleric acid (DL-APV, 100 μ M). GABA_A receptor mediated signalling was suppressed by picrotoxin (50 μ M) or bicuculline (20 μ M). The potassium channel blocker 4-Aminopyridin (4AP) was also used at 50 μ M on cortical slices. Drugs were obtained from Ascent Scientific (Bristol, UK).

Multi-unit activity and field potentials were recorded with up to four extracellular electrodes positioned with independent manipulators. Electrodes were made from formvar coated tungsten wire of diameter 50 μ m shaped electrolytically to a tip of diameter 5-10 μ m (Cohen and Miles (2000)). Extracellular potentials recorded between these electrodes and an Ag/AgCl ground were amplified 1000x by a four channel amplifier (AM systems M1700) using a pass band of 0.1 – 20 000 Hz. They were digitized with a 12 bit, A–D converter (Digidata 1200A, Molecular Devices) monitored with the program Axoscope (Molecular Devices) and saved to a computer.

A.5. Whole cell recordings

Organotypic cultures on glass coverslips were transferred to a chamber mounted on the stage of an Axioskop 2 FS plus microscope (Zeiss, France). They were perfused with an extracellular solution containing 124 mM NaCl, 2.5 mM KCl, 26 mM NaHCO₃, 1 mM NaH₂PO₄, 2 mM CaCl₂, 2 mM MgCl₂, and 11 mM D-glucose. This extracellular solution was equilibrated with 5% CO₂ in O₂ to maintain a pH near 7.3, osmolarity was kept in the range of 305-315 mOsm and heated to 32-34°C. Patch electrodes of resistance 3-6 M Ω , contained 130 mM K-gluconate, 5 mM KCl, 10 mM HEPES, 10 mM EGTA, 2 mM MgCl₂, 4 mM MgATP, 0.4 mM Tris-GTP and 10 mM Na₂-phosphocreatine. Neurons were visualized with EMCCD camera (Luca-S, Andor) using infrared differential interference contrast. An Axopatch 200A amplifier (Molecular Devices, USA) was used to make records in current clamp mode.

A.6. Trace analysis and statistics

Only records displaying ictal like and inter-ictal like activities were analysed.

For a better precision, the start and the end point of each activity were manually detected during 5 to 20 minutes on each interesting record. Analyses were made using Clampfit (Molecular Devices) and Prism software (Graphpad). Results were expressed by mean $\pm$

s.d. with significance of $P < 0.05$. The nonparametric Wilcoxon matched-pairs signed rank test were used to determine how significant was the pairing.

A.7. Anatomy

5 The morphology of organotypic cultures was compared with that of acutely prepared slices from the same patients. Tissue was fixed in 4 % PFA, washed in 0.1 M phosphate buffer (PB) and infiltrated with a sucrose-PB solution. Tissue was subsequently cryo-protected in a solution containing 15% sucrose and 30% polyethylene-glycol in PB and kept at -20°C. Sections were cut after washing in PB, and embedding in sucrose (15%)
10 and gelatine (7%). Sections of thickness 20 µm were cut on a freezing cryostat, mounted on superfrost slides and stored at -20°C.

 An antigen retrieval procedure was applied before immunohistochemical staining. Slides were washed in phosphate buffered saline (PBS), in a glycine solution (0.1 M in PBS, 5 min), and in a blocking solution (10% Donkey serum, 0.1% Triton-X in
15 PBS, 4 hrs at room temperature). Primary antibodies were applied in the same solution for 24h at 4°C, followed by washing overnight in PB at 4°C. The primary antibodies used were: anti-NeuN (monoclonal mouse; 1:200; Millipore), anti-GFAP (Chicken; 1:500), anti b-tubulin III (Mouse; 1:200), anti Nestin (Rabbit; 1:500). Secondary antibodies were then applied for 4hr in the same solution at room temperature. Secondary antibodies were: Cy-2
20 conjugated donkey anti chicken IgY (1:1000) anti mouse Cy3 and anti-rabbit CY5 (1:500, Jackson Immunoresearch, Baltimore, USA). After washing in PB for 4hr, slices were mounted with Prolong Gold antifade agent (Invitrogen).

A.8. Microscopy and image quantification

25 Images were acquired with an inverted microscope (Olympus IX81), an Optigrid II (QIopiQ) and camera (QImaging Retiga EXI) using an acquisition, scanning and measurement system (VLOCITY, Improvision, a perkin elmer company, Coventry, UK). The optigrid system permitted acquisition of structured images and subsequent 3D reconstruction. Stacks of images of organotypic slices were acquired with a 20x objective
30 of NA 0.85 (30-45 images at interval 0.7 µm with voxel size 0.64 µm) or with a 40x objective of NA 1.3 (40-70 images at 0.4µm with voxel size 0.32 µm).

A.9. Electron microscopy

For electron microscopy, acute slices were fixed overnight at 4°C in 4% paraformaldehyde and 0.25% glutaraldehyde in sucrose solution. The same fixatives were diluted in Hank's buffer solution. Slices were washed in PBS and postfixed in osmium tetroxide (1% in PBS, Uptima Interchim), dehydrated in graded ethanols, and embedded in Durcupan (Fluka). Images of semi thin and ultrathin sections of thickness 500 and 50 nm respectively, stained with uranyl acetate and lead citrate, were examined using a bright field or a transmission electron microscope (JEM 1010, JEOL).

B. RESULTS

Example 1: Activity in organotypic slices of an adult brain tissue sample cultured in an MEM-based or a novel defined medium according the instant invention ('OSCM' culture medium).

In initial experiments, multi-unit neuronal activity generated by slices of adult hippocampi (see "Materials and Methods" section for details) maintained in organotypic cultures according to Stoppini *et al.* (1991) or 'OSCM' (n=9 slices from 5 preparations). Multi-unit extracellular discharges were recorded from one of all tested region (the subiculum) in 1 to 4 trials at 6-15 days in culture with the medium according to Stoppini *et al.* (Fig. 1A). Synchronous bursts of multi-unit firing accompanied by field potentials were never detected. In contrast at 6-10 days in culture using an 'OSCM' culture medium, extracellular firing was detected in 21 of 23 cultured slices of the different regions tested: CA2/CA3, Dentate Gyrus, Subiculum. Epileptiform activity consisting of a burst of multi-unit firing of duration 20-100 ms accompanied by a field potential occurred spontaneously in 12 of 23 slices cultured in an 'OSCM' (Fig. 1B).

The probability of recording multi-unit (Fig. 1C) or epileptiform activity (Fig. 1D) was higher for organotypic slices cultured with the defined medium according to the invention as compared to the medium according to Stoppini *et al.*

Example 2: Time course of changes in epileptiform activities generated by organotypic slices of an adult brain tissue sample.

The activities generated by organotypic slices of an adult brain tissue sample were assessed over time in culture. The electrical activities of human epileptic slices were

recorded at 2-4, 6-10, 13-16 and 20-29 days in culture (Fig. 2 A-D). An electrical activity was recorded on 12 occasions from cultured slices prepared from the same tissue block. In all cases, epileptiform events were generated by either the CA3 region, the dentate gyrus or the subiculum. This site did not change in records made at different times in culture (n=39).

Records from the CA3 region revealed little spontaneous multi-unit discharges and no epileptiform activities at 2-4 days in culture. Interictal like activities were largely revealed after 6-10 days ($F=0.19 \pm 0.084$ Hz, n=5), increased after two weeks ($F=0.50 \pm 0.23$, n=3) then get back to the frequency observed after one week ($F=0.19 \pm 0.078$, n=3) (Fig 2A-D). Same variation in frequency were observed either on ACSF or in convulsant solution: respectively 0.57 ± 0.067 at d9, n=5; 1.36 ± 0.62 at d15, n=3; 0.71 ± 0.28 at 21d n=4 (Fig 3A). All results obtained from different region (CA2/CA3, Dentate Gyrus (DG), Subiculum) and after different times in culture, show that a significant difference in activities is observed between ACSF and convulsant solution: $F=0.18 \pm 0.033$ Hz versus $F=0.54 \pm 0.69$ Hz respectively, $P<0,001$ (Fig. 3B).

Example 3: Regional variation of activity at 6-10 days in culture.

Extracellular records were made of multi-unit neuronal and field potential activity from the hilus, the dentate gyrus (DG), the CA2-CA3 region, the CA1 region and the subiculum (n= 72 slices). At 6-10 days in culture, multi-unit activity at frequencies ranging between 0.5 and 200 spikes/sec was generated by at least one of these regions in 24 of 28 slices tested. Spontaneous multi-unit activity was recorded from the dentate gyrus in 13 of 16 slices, from the hilus in 5 of 8 slices, from the CA2-CA3 region in 11 of 14 slices and from the subiculum in 20 of 23 slices. We never detected multi-unit activity in records from the CA1 region of 3 slices.

Epileptiform activity consisting of several bursts of multi-unit firing with a local field potential was detected after 7-10 days in culture, in the dentate gyrus (7 of 16 slices), the CA2-CA3 region (6 of 14) and the subiculum (10 of 23 slices). Duration of spontaneous interictal-like discharges was 127.6 ± 52.6 ms for the DG, 59.7 ± 11.4 ms for the Subiculum, $67.7 \pm 18,6$ ms for the CA3 and recurred at interval 10.9 ± 3.8 s, 3.57 ± 0.6 s, 10.29 ± 4.47 s respectively. Local synchrony was confirmed in records of similar events at short latency from pairs of electrodes separated by 1-300 μ m. Comparing the

timing of events recorded in different regions suggested that activity could be initiated either in the dentate gyrus/ hilus (8 of 24 slices), in the CA2/3 region (6 of 14 slices) or in the subiculum (10 of 23 slices) and then spread to invade nearby regions (Fig. 4A). In 8 of 13 slices, distinct synchronous events appeared to be initiated in more than one region suggesting that several epileptic focus could occurs in a same time. Two distinct regions can also generate activities that mutually spread from one to the other as we observed between the CA3 and the DG.

Example 4: Pharmacological sensitivity and effects of convulsants on activity at 6-10 days in culture

Interictal-like activity generated by the subiculum in acute tissue slices from patients with temporal lobe epilepsies is blocked by antagonists at both glutamatergic and GABA_A receptors, suggesting that both receptors contribute to its generation. As shown in Fig. 5F, interictal like activity generated by organotypic slices was suppressed in 3 out of 3 experiments by the glutamate receptor antagonists NBQX (10 μ M) and DL-APV (50 μ M). Furthermore, spontaneous epileptiform activity was blocked (Fig. 5D) in 8 of 17 experiments by the GABA_A receptor antagonist picrotoxin (50 μ M) or bicuculline (20 μ M). Switching acute slices of human subiculum from ACSF to a “convulsant solution” (Mg⁺⁺ 0.2 mM, K⁺ 10 mM; n=8) was previously demonstrated to induce epileptiform activities. On organotypic human slices, convulsants start to increase frequency of interictal like activities in all tested regions (Fig. 5B, 3A) and typically spread to initiate firing in all areas of the tissue except the CA1 region. No synchronised firing pattern could be observed between activities generated in the CA2/3 region and the subiculum. Between each effector application, a wash out allows to restore the original firing pattern (Fig. 5C, E).

After long exposure of convulsant in 6/13 slices, recurring ictal-like events were generated with prolonged multi-unit firing and a stereotyped pattern of field potential bursts (Fig. 4B). They were initiated in the dentate (n=3) or the subiculum (n=3), and were of duration 31.63 ± 1.84 s for the dentate, 21.68 ± 3.7 s for the subiculum and recurred at interval of 68.29 ± 3.716 s, 58.19 ± 11.35 s respectively.

Example 5: Pharmacological modulation of cortical activity

Epileptiform activity may start to be generated by hippocampal tissue from healthy rats maintained in organotypic culture over several weeks possibly due to changes in synaptic connectivities. The electrical activity of hippocampal slices were compared with the electrical activity of temporal cortex that was resected prior to hippocampus removal.

Sparse or no spontaneous activities has been observed neither on MEM based medium (n=2) nor in “OSCM” (n=8) after one, two or three weeks of culture (Fig.6A, 6B, 6H). In extensive recordings, spikes activity at low-frequencies was detected in 1 of 3 cultured slices at 10 days of culture (Fig. 6B), prepared from 3 tissue blocks, but epileptiform activity was never detected. While neuronal activity was largely absent, “convulsant solution” always enhanced multi-unit discharges and often induced epileptiform activity (n=3/3) (Fig.6C). Same pattern of epileptiform activities were also obtained with the K⁺ channel blocker 4-AP (50 μ M; n=2/2) (Fig.6G). Between two pharmacological tests on the tissue, a wash out restored the silent activity observed in ACSF (Fig.6D, 6F). The use of the GABAergic inhibitor picrotoxin (50 μ M) on cortical slices, generates the opposite effect than the one observed on the subiculum (n=3/3) (Fig.6E): epileptiform activity appears with the same pattern as the one obtained with the “convulsant solution” or with 4-AP (Fig.6G).

Interictal like population bursts were never found on superficial layer but always in deep cortical layer 3 to 5, where pyramidal and granular cells are found. These activities generally spread over a distance up to 600 μ m along the four recording electrodes that were used. Nevertheless any ictal like activities was recorded on that tissue.

Example 6: Anatomy of organotypic cultures at 6-10 days in culture.

As observed in macroscopical view of an organotypic slice, the sclerotic area is still visible in CA1 and general aspect of the slice didn't change after several weeks in culture. The neuronal survival in different regions using immuno-staining experiments was also assayed. The neuronal lineage marker β -Tubulin III was used to stained neurons that are still present on the granular cell layer of the dentate gyrus at 8 days of culture, and it was observed that there is not a dramatic cell loss with time in culture. Glial cell are also present like on the CA3 regions at 15 days of culture as revealed by a GFAP staining. Very

few progenitor cells are present as revealed by the Nestin marker, showing that dividing cells don't invade and disorganise the tissue cell layers.

Example 7: Ultrastructure of organotypic cultures at 6-10 days in culture.

The state of intracellular organelles in organotypic cultures was assessed using electron microscopy by comparing semi-thin (500 nm) sections prepared at 6-10 days with freshly fixed temporal lobe tissue. The nuclei of neurons from cultures tended to have a round shape after culture compared to an irregular shape for nuclei of neurons from fresh tissue.

Transmission electronic microscopy observations on ultra-thin (50 nm) epileptic tissues sections permit to see active synapses containing transmitter vesicles, mitochondria and post synaptic density.

Example 8: Whole cell record from a subicular pyramidal cell in an organotypic slice.

Whole cell records were made from three neurones in the subiculum including that shown in Figures 7A–C.

Figures 7A and 7B show that depolarization, after applying a current pulse, elicits burst firing in subicular pyramidal cell. Figure 7C shows that a hyperpolarizing current injection reveals a sag consistent with the presence of the hyperpolarization-activated (I_h) current.

In conclusion, these cells discharged in stereotyped bursts of action potentials as do some neurones of rodent and epileptic human subiculum.

REFERENCES

Armentano *et al.* (2011) Culturing conditions remarkably affect viability and organization of mouse subventricular zone in ex vivo cultured forebrain slices. *J Neurosci Methods*. 197:65-81.

Bonnet *et al.* (1998) CO₂/HCO₃⁻ withdrawal from the bath medium of hippocampal slices: biphasic effect on intracellular pH and bioelectric activity of CA3-neurons. *Brain Research* 796:161-70.

Brewer *et al.* (1993) Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. *J Neurosci Res.* 35: 567-76.

Chaichana *et al.* (2007) Preservation of glial cytoarchitecture from ex vivo human tumor and non-tumor cerebral cortical explants: A human model to study neurological diseases. *J Neurosci Methods.* 164: 261-70

Cohen and Miles (2000) Contributions of intrinsic and synaptic activities to the generation of neuronal discharges in in vitro hippocampus. *J Physiol.* Apr 15;524 Pt 2:485-502.

Gähwiler (1988) Organotypic cultures of neural tissue. *Trends Neurosci.* 11: 484-9.

Gähwiler *et al.* (1997) Organotypic slice cultures: a technique has come of age. *Trends Neurosci.* 20: 471-7

González-Martínez *et al.* (2007) Neurogenesis in the postnatal human epileptic brain. *J Neurosurg.* 107: 628-35.

Hansen *et al.* (2010) Neurogenic radial glia in the outer subventricular zone of human neocortex. *Nature.* 464: 554-561.

Jung *et al.* (2002) Brain tumor invasion model system using organotypic brain-slice culture as an alternative to in vivo model. *J Cancer Res Clin Oncol.* 128: 469-76

Kleinberger-Doron and Schramm. (1990) Culture of mature hippocampus slices for 4 days in a newly developed medium: preservation of transmitter release and leucine incorporation into protein. *Brain Res.* 533: 239-47

Stoppini *et al.* (1991) A simple method for organotypic cultures of nervous tissue. *J Neurosci Methods.* 37: 173-82.

Wilhelmi *et al.* (2002) Organotypic brain-slice cultures from adult rats: approaches for a prolonged culture time. *Altern Lab Anim.* 30: 275-83.

CLAIMS

1. Nutrient medium of defined composition for the *in vitro* organotypic culture of an adult brain tissue sample, said nutrient medium comprising (i) one or more pH
5 buffering system(s); (ii) one or more inorganic salt(s); (iii) one or more trace element(s); (iv) one or more free amino acid(s); (v) one or more vitamin(s); (vi) one or more hormone(s); (vii) one or more carbon/energy source(s); wherein said nutrient medium presents an osmolarity of from about 290 to about 330 mOsm, preferably of from about 300 to about 320 mOsm, most preferably of about 310 mOsm; wherein said nutrient
10 medium has a pH of from about 7.0 to about 7.4, preferably of from about 7.1 to about 7.3, most preferably of about 7.2; and wherein said nutrient medium presents a glutamine content and a glutamic acid content of from about 1 to about 250 μ M, preferably of from about 1 to about 150 μ M, most preferably of from about 5 to about 20 μ M.

2. Nutrient medium according to claim 1, wherein one or more free amino
15 acid(s), with the exception of glutamine and glutamic acid, is/are comprised at a concentration ranging from about 0.1 μ M to about 2,000 μ M, preferably at a concentration ranging from about 1 μ M to about 1,000 μ M, most preferably at a concentration ranging from about 5 μ M to about 250 μ M.

3. Nutrient medium according to any of the preceding claims, wherein one or
20 more vitamin(s) is/are selected in a group comprising biotin (vitamin H); D-calcium-pantothenate; choline chloride; folic acid (vitamin B9); myo-inositol; nicotinamide; pyridoxal (vitamin B6); riboflavin (vitamin B2); thiamine (vitamin B1); cobalamine (vitamin B12); acid ascorbic; α -tocopherol (vitamin E) and a combination of two or more vitamins thereof.

25 4. Nutrient medium according to any of the preceding claims, wherein one or more hormone(s) is/are selected in a group comprising insulin; 17 β -estradiol; human transferrin; progesterone; corticosterone; triiodothyronine (T3) and a combination of two or more hormone(s) thereof.

5. Nutrient medium according to any of the preceding claims, wherein one or
30 more carbon/energy source(s) is/are selected in a group comprising D-glucose; pyruvate; lactate; ATP; creatine; creatine phosphate and a mix thereof.

6. Nutrient medium according to any of the preceding claims, wherein it comprises at least two carbon/energy sources, said carbon/energy sources comprising lactate and pyruvate, and wherein lactate and pyruvate are preferably present in a weight ratio lactate/pyruvate of from about 15 to about 25, preferably of from about 17 to about 21, most preferably of about 19.

7. Nutrient medium according to any of the preceding claims, wherein it may also comprise at least an additional compound, preferably selected in a group comprising oxaloacetic acid; glutathione; glycerol; acetic acid; citric acid; thymidine; lipoic acid; linoleic acid; hypoxanthine; β -hydroxybutyrate and a mix thereof.

8. Nutrient medium according to any of the previous claims, wherein it may also comprise at least an antioxidant, preferably selected in a group comprising putrescine; superoxide dismutase (SOD); ascorbic acid; citric acid; acetic acid; glutathione; selenium and a mix thereof.

9. Nutrient medium according to any of the preceding claims, wherein it may also comprise at least a neurotransmitter and their precursor, preferably selected in a group comprising GABA, glutamate; glycine; aspartate; histidine; tryptophane; tyrosine; choline; phenylalanine and a mix thereof.

10. Nutrient medium according to any of the preceding claims, wherein said nutrient medium is serum-free.

11. Nutrient medium according to any of the preceding claims, wherein the pH buffering system is HEPES-free.

12. Use of a nutrient medium according to any of claims 1 to 11 for the *in vitro* organotypic culture of an adult brain tissue sample.

13. Use according to claim 12, wherein the adult brain tissue sample is an adult hippocampus and/or temporal cortex tissue sample.

14. Use according to any of claims 12 and 13, wherein the adult brain tissue sample originates from a human individual presenting an epileptic condition.

15. Method for *in vitro* maintaining a neuronal and a glial cellular activity of an adult brain tissue sample comprising a step of incubating said adult brain tissue sample in said nutrient medium according to any of claims 1 to 11 in conditions allowing survival of neuronal and glial cells.

A



B

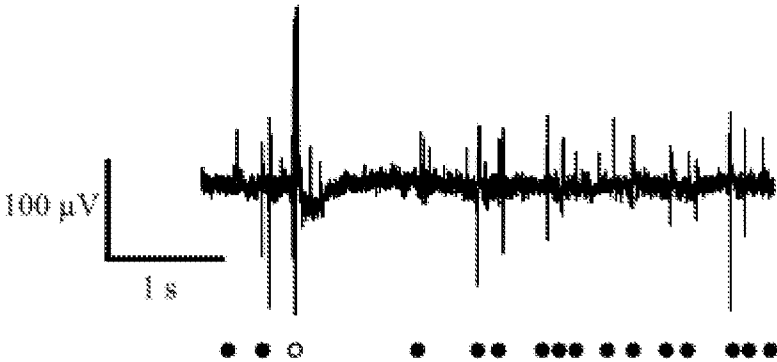
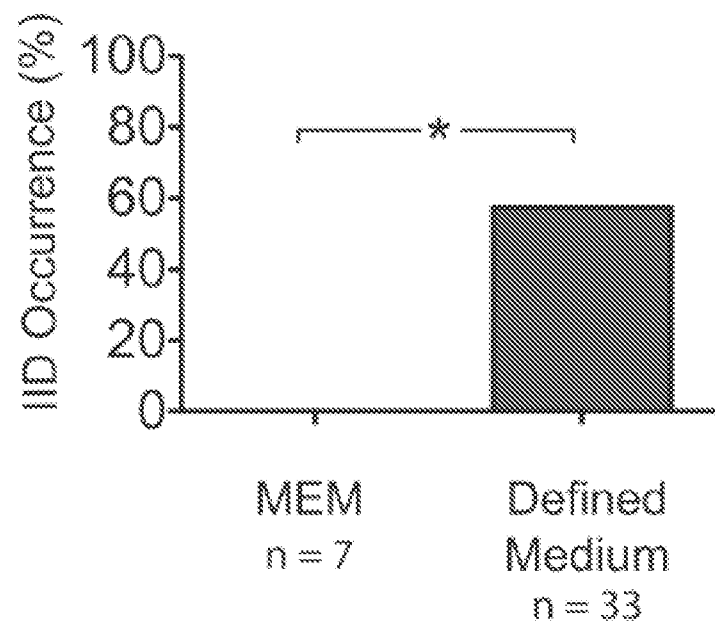


FIGURE 1

C



D

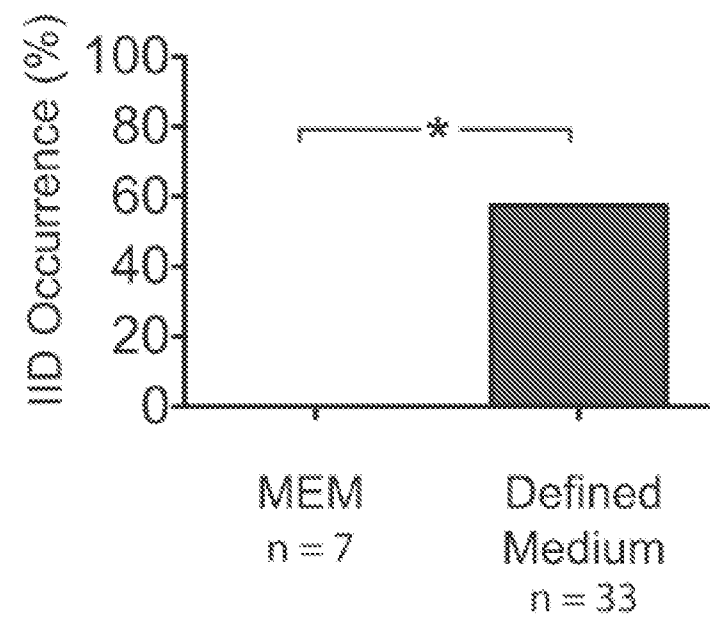


FIGURE 1

A



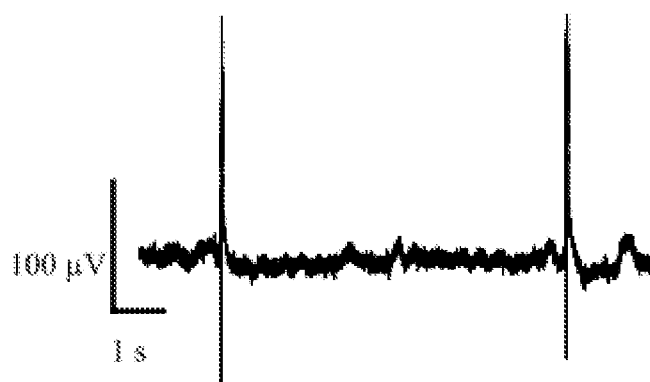
B



C



D

**FIGURE 2**

A



B

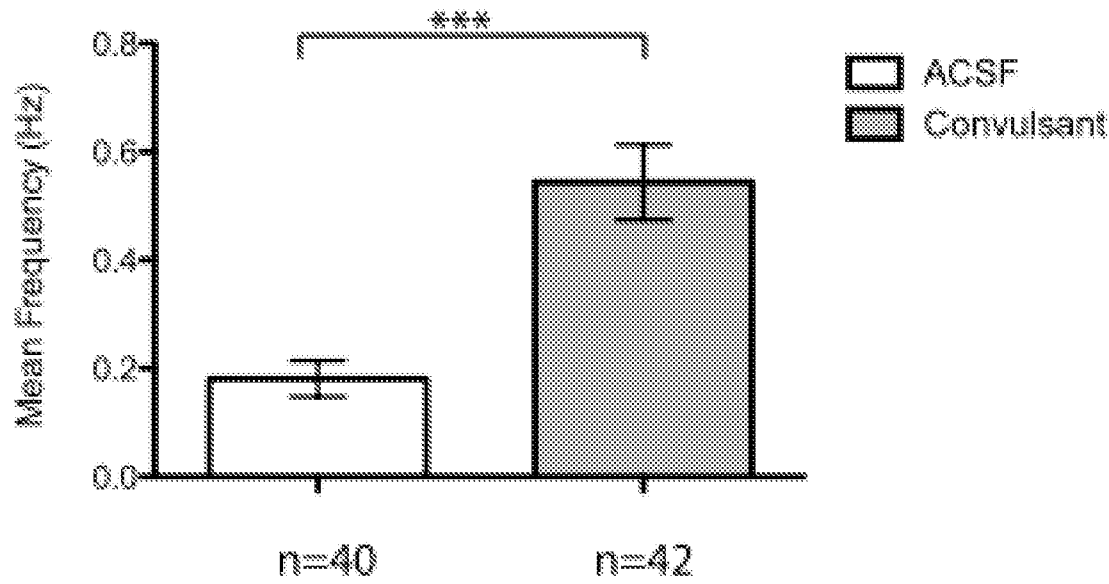
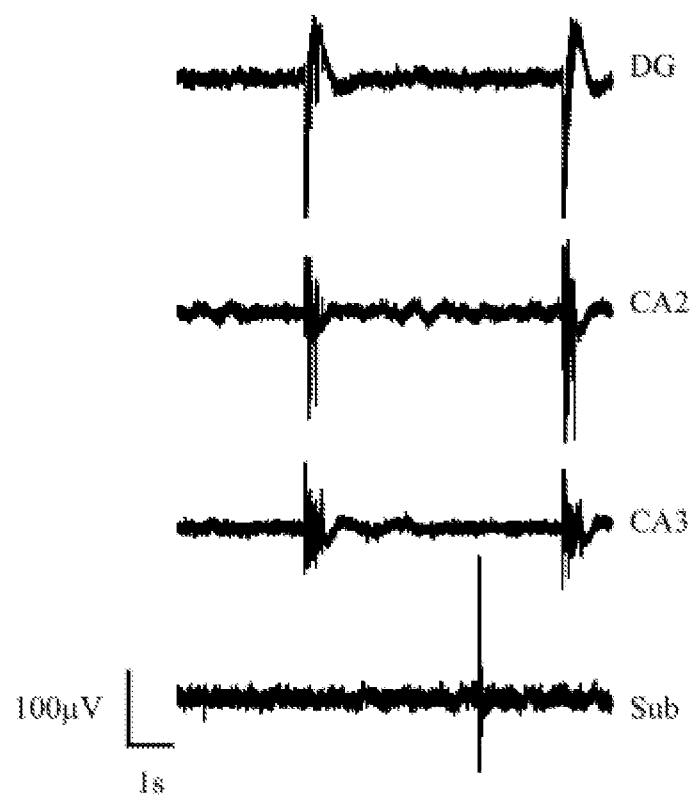
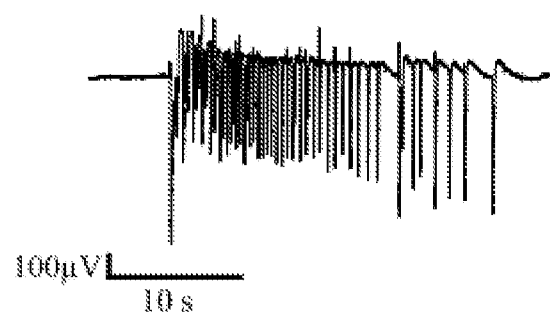
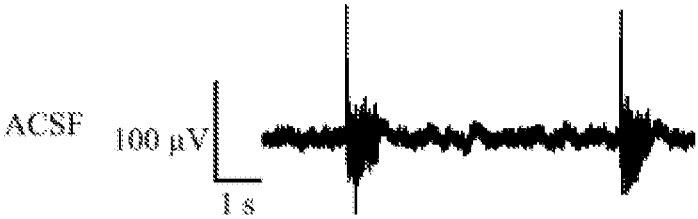


FIGURE 3

A**B****FIGURE 4**

A



B



C



D



E



F



FIGURE 5

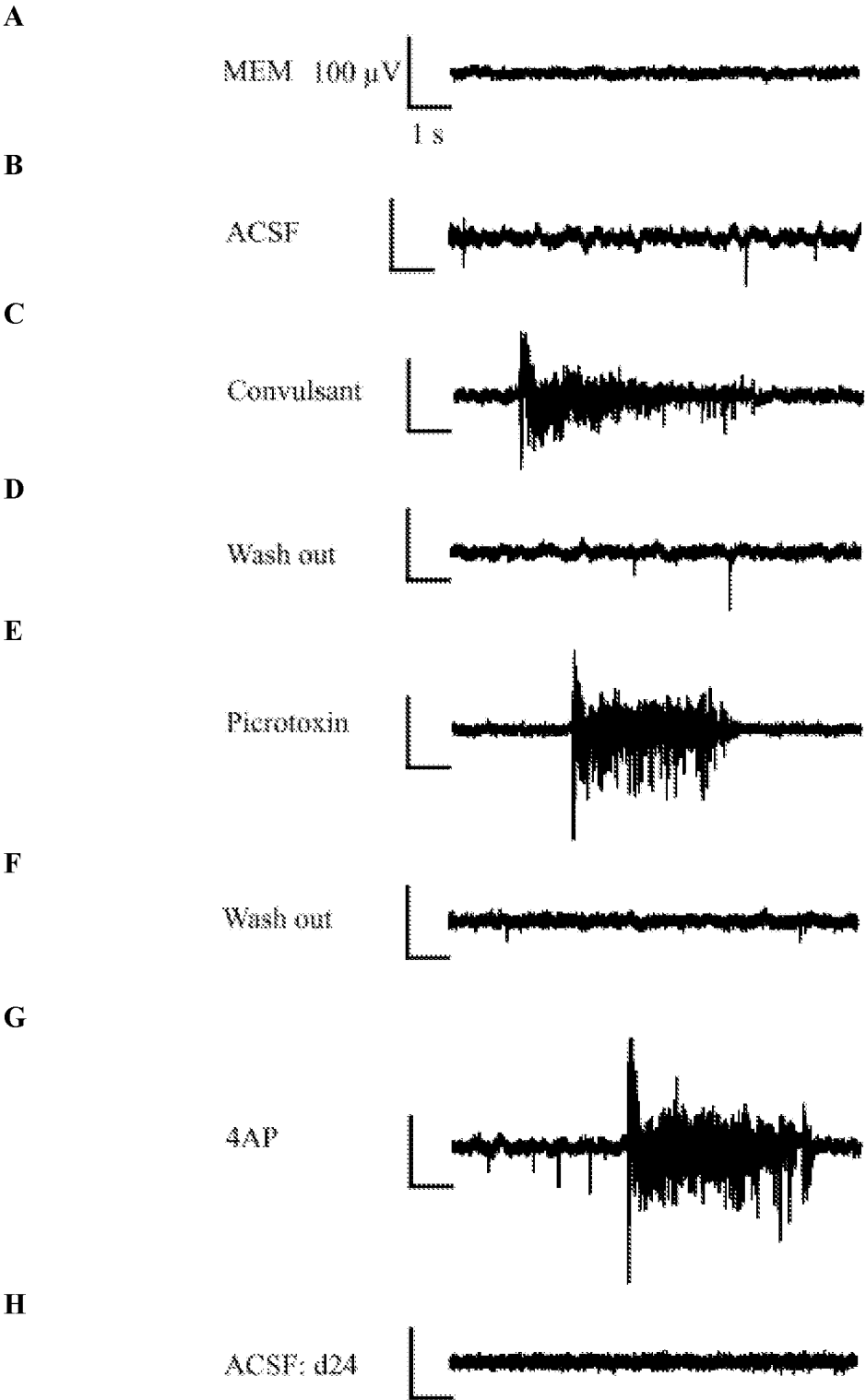
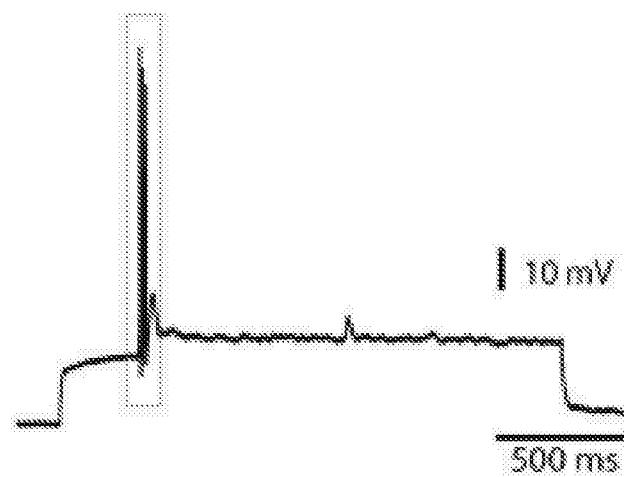
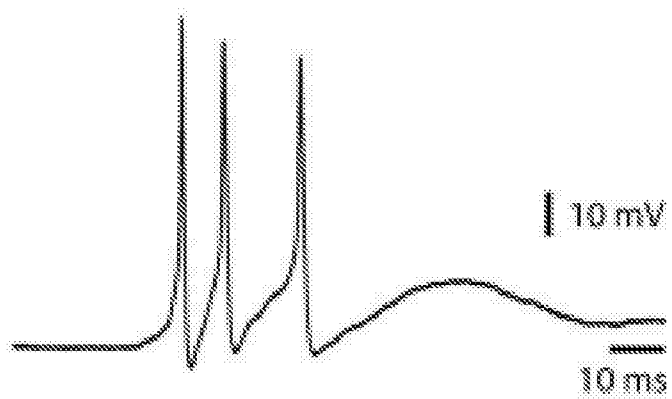


FIGURE 6

A



B



C

**FIGURE 7**

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2014/066393

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N5/079 C12N5/0793
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2012/052577 A1 (ESPINOSA DE LOS MONTEROS MARIA DOLORES ARACELI [US] ET AL) 1 March 2012 (2012-03-01) abstract paragraphs [0003], [0004], [0009], [0010], [0016], [0018], [0022], [0023], [0024] -----	1-15
X	US 4 657 866 A (KUMAR SUDHIR [US]) 14 April 1987 (1987-04-14) abstract column 1, lines 10-22 tables 1,2 claims 1, 7 ----- -/-	1-15



Further documents are listed in the continuation of Box C.



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Date of the actual completion of the international search

5 February 2015

Date of mailing of the international search report

12/02/2015

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer

Chavanne, Franz

INTERNATIONAL SEARCH REPORT

International application No

PCT/IB2014/066393

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 910 443 A (COON HYDEN G [US]) 8 June 1999 (1999-06-08) abstract column 5, lines 8-17 table 1 -----	1-15
X	BREWER GREGORY J ET AL: "Effect of Neuregen nutrient medium on survival of cortical neurons after aspiration lesion in rats.", JOURNAL OF NEUROSURGERY, vol. 98, no. 6, June 2003 (2003-06), pages 1291-1298, XP009176338, ISSN: 0022-3085 abstract page 1292, column 1, paragraph 2 -----	1-15

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/IB2014/066393

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
US 2012052577	A1	01-03-2012	NONE	
US 4657866	A	14-04-1987	NONE	
US 5910443	A	08-06-1999	NONE	