Diploma thesis

Organotypic slice cultures of the adult rat brain

An approach for improved cell viability

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Vienna, 7th March 2023

Declaration of Academic Integrity

I hereby confirm that the present diploma thesis is the result of my own independent scholarly work. I also confirm that in all cases, where material from the work of others (in books, articles, essays, dissertations, and on the internet) is acknowledged, quotations and paraphrases are clearly indicated. No material other than that cited in the reference list has been used. I have read and understood the Medical University's regulations and procedures concerning plagiarism.

Vienna, 7th March 2023 *Lennart Tögl m.p.*

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Table of contents

Acknowledgements 2
Table of contents 3
Abbreviations 5
List of figures 6
List of tables 7
Zusammenfassung 8
Abstract 10
1 Introduction 11
1.1 Organotypic cultures as a method in neuroscience 11
1.1.1 History and development 11
1.1.2 Slice cultures of specific brain regions 13
1.1.3 Co-cultures 14
1.2 Advantages and disadvantages of organotypic cultures 14
1.3 Applications in disease models (examples) 15
1.4 Assessment of viability in brain slice cultures and viability in neonatal vs. adult brain organotypic cultures 17
1.5 Culture media 21
1.5.1 Promising medium formulations and supplements for adult organotypic brain cultures 21
1.5.1.1 Serum-free media 21
1.5.1.2 Insulin 22
1.5.1.3 Ascorbic acid 23
1.5.1.4 Growth factors 23
1.6 Unanswered questions 24
1.7 Aims and hypotheses 24
2 Materials and Methods 25
2.1 Materials 25
2.2 Methods 27
2.2.1 Tissue harvest 27
2.2.2 Slice preparation and cultivation 27
2.2.3 Staining 28
2.2.4 Microscopy 28
2.2.5 Slice exclusion 29
2.2.6 Data analysis and quantification 29
2.2.7 Statistical analysis 30
3 Results 32
3.1 Total cell counts 32
3.1.1 Day 1 32
3.1.2 Day 3 33
3.1.3 Day 5 34
3.1.4 Day 7 35
3.1.5 Summary of total cell counts 36
3.2 Cortex 37
3.2.1 Day 1 37
3.2.2 Day 3 38
3.2.3 Day 5 39
3.2.4 Day 7 39
3.2.5 Summary cortex 40

3.3 Hippocampus 41
3.3.1 Day 1 41
3.3.2 Day 3 42
3.3.3 Day 5 43
3.3.4 Day 7 44
3.3.5 Summary hippocampus 44
3.4 Thalamus 46
3.4.1 Day 1 46
3.4.2 Day 3 47
3.4.3 Day 5 48
3.4.4 Day 7 49
3.4.5 Summary thalamus 50
4 Discussion 51
4.1 Serum-free NB-A and serum-containing BME 52
4.2 Insulin 53
4.3 Ascorbic acid 54
4.4 Ascorbic acid in combination with insulin 54
4.5 Limitations 55
4.6 Outlook, recommendations, and conclusion 56
5 References 57
Appendix 69

Abbreviations

AA	ascorbic acid, vitamin C
ANOVA	analysis of variance
BDNF	brain-derived neurotrophic factor
BME	Basal Medium Eagle
calcein-AM	calcein acetoxymethyl ester
DAPI	4′,6-diamidino-2-phenylindole
DMSO	dimethyl sulfoxide
DNA	desoxyribonucleic acid
D-PBS	Dulbecco's Phosphate Buffered Saline
EthD-1/2	ethidium homodimer-1/2
FOV	field of view
GABA	γ-aminobutyric acid
HBSS	Hank's Balanced Salt Solution
HS	horse serum
LDH	lactate dehydrogenase
MEM	Eagle's Minimum Essential Medium
NADH	reduced nicotinamide adenine dinucleotide
NB-A	Neurobasal A medium
NGF	nerve growth factor
OTC	organotypic (slice) culture
PBS	phosphate-buffered saline

List of figures

Figure 1: Setup of organotypic tissue culture in Maximow assemblies. 12
Figure 2: Day 1 (total), viability by medium. 33
Figure 3: Day 3 (total), viability by medium. 34
Figure 4: Day 5 (total), viability by medium. 35
Figure 5: Day 7 (total), viability by medium. 36
Figure 6: Cortex day 1, viability by medium. 37
Figure 7: Cortex day 3, viability by medium. 38
Figure 8: Cortex day 5, viability by medium. 39
Figure 9: Cortex day 7, viability by medium. 40
Figure 10: Cortical tissue on day 1 in-vitro. 41
Figure 11: Hippocampus day 1, viability by medium. 42
Figure 12: Hippocampus day 3, viability by medium. 43
Figure 13: Hippocampus day 5, viability by medium. 44
Figure 14: Hippocampus day 7, viability by medium. 44
Figure 15: Hippocampal tissue on day 1 in-vitro. 46
Figure 16: Thalamus day 1, viability by medium. 47
Figure 17: Thalamus day 3, viability by medium. 48
Figure 18: Thalamus day 5, viability by medium. 49
Figure 19: Thalamus day 7, viability by medium. 50
Figure 20: Thalamic tissue on day 1 in-vitro. 51

List of tables

Table 1: Utilized statistical tests by group and their results. 69
Table 2: Total day 1 post-hoc test following significant Welch's ANOVA. 70
Table 3: Kruskal-Wallis' test for total day 1 with Bonferroni correction (15 comparisons). 71
Table 4: Total day 3 post-hoc test following significant ANOVA. 72
Table 5: Kruskal-Wallis' test for total day 3 with Bonferroni correction (15 comparisons). 73
Table 6: Cortex day 1 post-hoc test following significant ANOVA. 74
Table 7: Cortex day 3 post-hoc test following significant ANOVA. 75
Table 8: Kruskal-Wallis' test for cortex day 3 with Bonferroni correction (15 comparisons). 76
Table 9: Kruskal-Wallis' test for hippocampus day 3 with Bonferroni correction (26 comparisons). 76
Table 10: Thalamus day 1 post-hoc test following significant ANOVA. 77
Table 11: Kruskal-Wallis' test for thalamus day 3 with Bonferroni correction (15 comparisons). 78

Zusammenfassung

Einführung: Die organtypische Kultur (OTC) ist eine vielversprechende Methode in den Neurowissenschaften, bei der Hirngewebe neugeborener Versuchstiere typischerweise in Schnittform in vitro kultiviert wird. In adulter OTC sinkt die Anzahl lebender Zellen kontinuierlich. In der Forschung über adulte Hirnerkrankungen findet sie daher keine breite Anwendung, weshalb verbessertes Zellüberleben erstrebenswert ist. Es gibt Hinweise auf Vorteile serumfreier Zellkulturmedien (Neurobasal A, NB-A), von Insulin und Ascorbinsäure (AA). Das Forschungsziel dieser Arbeit ist die Beurteilung, ob NB-A, Insulin, AA oder Insulin und AA in Kombination einen überlebensfördernden Effekt auf das gesamte Gehirn, Kortex, Hippocampus oder Thalamus haben.

Methoden: Adulte Ganzhirnschnitte 9-12 Wochen alter Ratten wurden mit 1) klassischem Medium mit Serum (BME), 2) BME + Insulin, 3) BME + AA, 4) BME + Insulin + AA, 5) NB-A, 6) NB-A + Insulin, 7) NB-A + AA, 8) NB-A + Insulin + AA kultiviert. Nach jeweils 1, 3, 5 und 7 Tagen wurde das Gewebe mit Calcein (markiert lebende Zellen) und EthD-1 (tote Zellen) angefärbt, und Bilder mit einem konfokalen Laserrastermikroskop aufgenommen. Zellen wurden in Cortex, Hippocampus und Thalamus mittels BoneJ-Plugin der Bildbearbeitungssoftware FIJI gezählt und der Prozentsatz lebender Zellen bestimmt. Zusätzlich wurde die Überlebensrate des gesamten Hirns rechnerisch ermittelt. Die Nährmedien wurden durch ANOVA und Kruskal-Wallis-Test miteinander und mit einer Totkontrolle verglichen.

Ergebnisse: An Tag 3 war die gesamte und kortikale Überlebensrate von mit NB-A kultivierten Schnitten im Vergleich zu BME signifikant besser. Generell unterschieden sich die Gruppen mit Insulin, Ascorbinsäure oder beidem nicht signifikant von den Gruppen ohne sie, aber alle Zusätze könnten vorteilhaft für bestimmte Gewebe zu bestimmten Zeitpunkten sein. Insulin + AA zeigte eigenständige Effekte, die es von Insulin und AA unterschieden.

Diskussion: Weitere Studien über diese Nährmedien und Zusätze sind sinnvoll, aber zusätzliche Maßnahmen scheinen nötig zu sein, um die Zellüberlebensrate deutlich zu erhöhen. Hochspezifische, an Hirnregion und Zeitpunkt angepasste Nährmedien, sowie die Optimierung anderer Umweltfaktoren, können die nächsten logischen Schritte sein. In dieser Arbeit sind folgende Einschränkungen zu beachten: Die Calcein/EthD-1-Färbung kann nicht zwischen Zelltypen unterscheiden, die Stichproben waren klein

unterschiedlich groß, außerdem traten Herausforderungen bei der digitalen Zellerkennung sowie doppelt angefärbte Zellen auf.

Abstract

Introduction: Organotypic slice culture of the brain (OTC) is a valuable technique in neuroscience. OTC slices are commonly tissues from neonatal donor animals. In adult slices, cell viability continuously drops - reason for the lack of its widespread application to studies on adult brain pathologies. Hence, improvement of viability is desirable. Reports point to potential benefits of non-classical, serum-free culture medium formulations (Neurobasal A, NB-A), insulin and ascorbic acid (AA) in adult OTC. The aim of this study was to assess if NB-A, the supplementation of insulin, AA or the combination of the last two can better retain live cells in whole-brain OTC or in cortical, hippocampal, or thalamic tissue therein.

Methods: Adult whole-brain slice cultures prepared from 9–12-week-old rats were treated with 1) a serum-containing medium (BME), 2) BME + insulin, 3) BME + AA, 4) BME + insulin + AA, 5) NB-A, 6) NB-A + insulin, 7) NB-A + AA, 8) NB-A + insulin + AA. On days 1, 3, 5 and 7 in-vitro, after labelling with calcein (live cells) and EthD-1 (dead cells), confocal laser scanning microscopy images were taken, and cells counted by BoneJ plugin in FIJI image processing software in cortex, hippocampus, and thalamus. A total cell count was calculated. Experimental groups and dead control were compared by one-way ANOVA or Kruskal-Wallis' test.

Results: Neurobasal A medium was significantly better than BME on day 3 in total cell counts and cortex. Generally, groups treated with insulin, AA or both did not significantly differ from those without them, but all supplements could be beneficial for certain tissues at certain timepoints. Insulin + AA supplement had distinct effects other than insulin or AA.

Discussion: The investigated media and supplements are interesting for further investigation but might require additional measures to improve viability. Tailoring highly specific media optimized to brain region and time point of interest or considering other environmental factors is advisable. In this study, the following important limitations apply: Calcein/EthD-1 staining cannot discriminate between cell types, sample size differs between groups and is small. Double-staining and challenges in digital cell location occurred.

1 Introduction

1.1 Organotypic cultures as a method in neuroscience

Organotypic (slice) culture (OTC) is a method in biomedical science that represents a link between in-vitro and in-vivo experiments. (1) In-vitro experiments, on the one hand, are a widespread technique (1) usually performed in a petri dish or a similar container with a single cell type, as for example human cervical cancer cells (HeLa-cells). In this manner, the cells are relatively easy to access and to perform experiments on. (2) However, they are less accurate when it comes to mimicking conditions in an organism, (1) as they are devoid of, for instance, tissue architecture, innervation, continuous blood supply etc. In-vivo experiments, on the other hand, are animal experiments conducted with and in a living organism comprised of many different cells, tissues, and organs, and have a higher translational value. (3) In present-day organotypic cultures, organs usually obtained from rodents like rats or mice are explanted and subsequently thinly sliced. These tissue slices are then cultivated under culture conditions. (4) Tissue slices allow for the study of complete, complex tissues – such as the brain, or human testicular tissue, (5) that enable to study interactions between different cell types maintaining their distinct tissue structure in-vitro. Thus, they represent a closer approximation to what happens in a living organism than more commonly studied isolated cells. (6) However, they are not as time-consuming and expensive as animal experiments. (7)

If not mentioned otherwise, this work will focus on organotypic cultures of rodent brain tissue.

1.1.1 History and development

The long-term organotypic culture technique was predated by tissue explants. (1) The earliest precursor of today's brain organotypic culture technique was the ex-vivo study of embryonic frog tissue in the early 20th century in hanging drop position. (8,9) A coverslip was coated with adult frog lymph and embryonic frog ectoderm tissue was placed onto it. After the lymph had clotted, a hollow second coverslip was placed onto the first one, sealed to prevent from drying and placed upside down. (8,10) This technique enabled the observation of developing nervous tissue over weeks. It was later modified for other tissues. (10)

As reviewed by Crain, Maximow defined the two terms „organotypic“ and „histiotypic“ for the growth behaviour of cultured tissues in 1925: Histiotypic relates to the disseminated growth of cells outwards from a cut surface, whereas organotypic describes a rather organized situation that – according to Fell – includes maintained tissue architecture, function as well as maturation of still immature tissue in-vitro reaching a state similar to in-vivo adult tissue. (compare to this section (9))

As reviewed by Schwerdtfeger, the Maximow method was implemented and used for the culture of, for example, embryonic tissue in the early 20th century. (10) The tissue was placed on a rectangular, collagen-coated coverslip (alternatively, blood plasma was used) with a drop of medium. A bigger glass slide with a central concavity was placed over the tissue and the whole assembly was then sealed with paraffin jelly. The loaded coverslips were then stored in lying-drop position with the tissue facing upwards. If microscopy of the tissue was desired, it was flipped upside down for microscope access (hanging drop position). This method required the investigators to take great care in disassembling the coverslip and the glass slide to allow for feeding of the culture with a drop of fresh culture medium (Figure 1). (compare to this section (10,11))

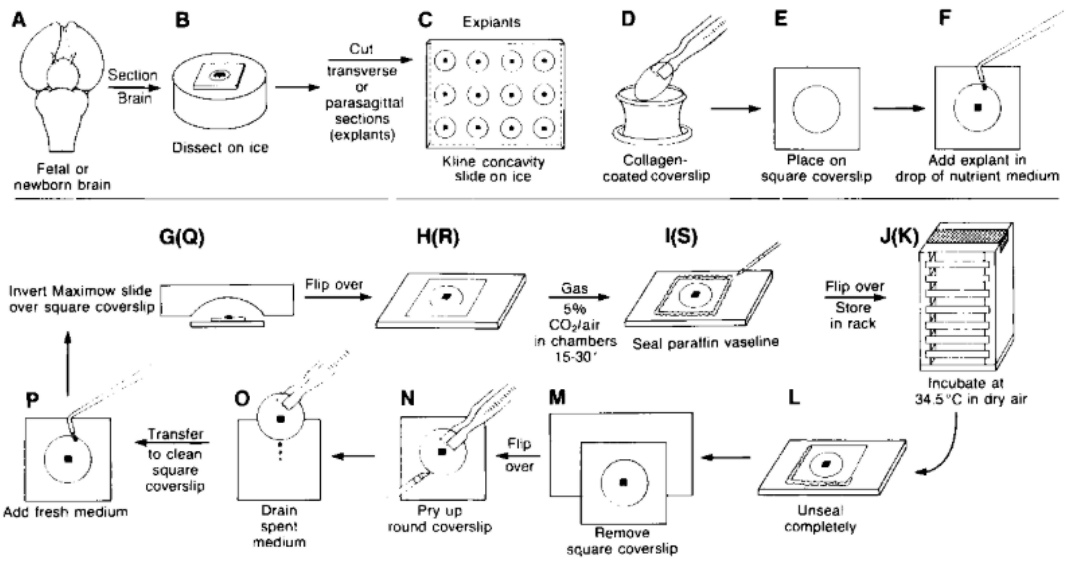

Figure 1: Setup of organotypic tissue culture in Maximow assemblies.
Please refer to source (11) and the appendix for the license.

In the 1950s brain slicing was introduced, (12) e.g. for the study of resting potential in mammalian brain tissue. (13) From then on, many studies made use of reproducible brain

slicing methods. (10) At this time, most experiments with brain slices were conducted only within a few hours. (10) Today, this type of brain slices is referred to as acute slices. (6)

As reviewed by Alaylioğlu et al., Hogue introduced the roller tube-technique, which Gähwiler further optimized in the 1980s. (1) When using this technique, researchers place the sliced tissue on a coverslip and embed it in a plasma drop, which subsequently coagulates. Alternatively, it is embedded in a collagen matrix. Then, the coverslip with the affixed tissue is stored in a medium-containing tube in a roller drum inside an incubator. The slow, constant rotation submerges the tissue in the culture medium half of the rotation period and thus ensures ventilation, in this manner providing both nutrients and oxygen to the tissue. Due to the rotation, the tissue flattens up to a thickness of 50µm for an original slice thickness of 400µm and eventually nearly becomes a single cell layer, which makes the roller-tube technique useful for single cell microscopy or investigations requiring high access to individual cells. By means of the roller-tube technique, tissue can be cultivated in-vitro for up to several months. (compare to this section (14))

Stoppini and his group finally introduced the membrane-interface technique for organotypic cultures, in which organ slices are transferred on a semipermeable membrane. From beneath, the membrane and the tissue are wetted by a cell culture medium that supplies the tissue with nutrients, whereas oxygen is delivered to the tissue by the air from above. Membrane-interface OTCs do not flatten as much as roller tube OTCs and therefore retain their three-dimensional tissue structure better. (compare to this section (4))

Both methods are suitable for a variety of methods, e.g., electrophysiology. Membrane-interface slices are easy to handle, and, in contrast to the roller-tube slices, more apt for chemical treatment, drug testing or gene overexpression experiments as the tissue is not coated by medium. Hence, this method prevails today. (compare to this section (1))

1.1.2 Slice cultures of specific brain regions

Typically, tissue for brain slice cultures is obtained from one specific brain region. A wide range of regions have been used for this purpose, as for instance hypothalamus, locus coeruleus, raphe, basal forebrain, suprachiasmatic nucleus and supraoptic nucleus, olfactory system, (6,15–20) cerebellum, hippocampus, cortex, thalamus, striatum, substantia nigra; the most commonly used regions are hippocampus and cerebellum. (1) Furthermore, several groups reported the preparation of whole brain slice cultures, which

are typically cut either sagittally (21) or coronally. (22) Whole-brain slices were claimed to be less susceptible to cell death, as more projections to neurons in other brain regions are kept. (23)

1.1.3 Co-cultures

A variant of OTCs are co-cultures, with which two or more brain regions can be cultivated together at the same time. This is especially interesting for the study of a whole connected system in the brain, as for instance the striatonigral system in research about Parkinson's disease. (6) An interesting application of organotypic co-cultures is the recreation of the blood-brain barrier in-vitro utilizing, for example, a brain slice and endothelial cells. (24)

1.2 Advantages and disadvantages of organotypic cultures

In contrast to classic cell culture methods working with only one cell type, the study of complex interactions between different cell types and tissues can be realized in-vitro using organotypic slice cultures. In addition, the three-dimensional structure of the brain is partly retained. This makes OTCs a closer approximation of what happens in a living organism. Depending on the region of interest within the central nervous system, brain OTCs can comprise of different neuronal populations, glial cells like astrocytes, oligodendrocytes, ependymal cells, microglia as well as blood vessels. Furthermore, the application of OTCs is suited to reduce the number of laboratory animals in research, as many slices can be obtained from a single brain. (compare to this section (6)) In addition, this might decrease variability between single slices caused by the utilization of tissue from several individual animals. (7) What is more, OTC tissue is easily accessible to observation (i.e. microscopy) and manipulation. (4)

When working with OTCs, it must be taken into account that only brain regions with multiple cell strata are suitable for slice culturing, as only these brain regions can be cut in plane without extensive damage of the contained axons, which is known to cause neuronal cell death. (25) In some studies, however, this axotomy-related cell death might even be desirable. (26) If tissue is obtained from mature animals, it will be more susceptible to deleterious influences (27,28) and its cultivation is more challenging than of postnatal tissue. (6) As the slicing disrupts the already formed neuronal network in-vivo, it is naturally demanding for the adult tissue to recover in-vitro. In contrast to adult tissue, neonatal tissue is able to stabilize viability over time. (26) Furthermore, the slice

preparation process is technically challenging and demands well-practiced manual skills. (1) As for OTCs prepared with the roller-tube technique, their preparation is very time-consuming and extensive changes in tissue architecture through migration of glial cells occur. (25) In membrane-interface OTCs less derangement of tissue structure occurs, but glial cells tend to multiply on the surface and may hinder penetration of desired substances into the tissue. (25) In addition, vessel structures in OTCs are not operational and cannot transport e.g., oxygen, nutrients or hormones to and from the tissue. Without an operational vessel system, pharmacokinetic studies are not feasible with classical OTC methodology. (1)

1.3 Applications in disease models (examples)

Organotypic brain slice culture is a versatile method applicable to a wide variety of research questions. In this section, a few non-exhaustive examples of OTC as a method to model human diseases in-vitro will be presented.

OTC can be a useful method in experimental oncology. One possibility to employ OTCs in this research area is to prepare organotypic slices of tumour tissue itself. Such a model was, for example, used to study cell migration within human glioblastoma tissue. (29) Another interesting application of OTCs is tumour cell seeding on healthy brain tissue to study tumour formation and metastasis. (30)

OTCs have been a useful tool in Alzheimer's Disease research. Several groups investigated beta-amyloid toxicity as a feature of the disease in hippocampal slices. For instance, they examined apoptosis, intracellular pathways, and neuroprotection. (6,31–33)

The striatonigral system - which is affected in Parkinson's Disease through cell death of dopaminergic neurons in the substantia nigra (34) - is well established in organotypic slice cultures with several studies focusing on this part of the brain. (6) A common method to induce cell death of dopaminergic neurons is the use of neurotoxins. (35) In contrast, in another attempt to mimic the pathology of Parkinson's disease, the mere use of slicing inherent to OTCs has been employed to induce dopaminergic neuronal cell death. (36)

In Multiple Sclerosis research, OTCs were applied, for instance, to investigate demyelination and remyelination in both postnatal and adult cerebellar slices. The demyelination can hereby be induced using lysolecithin. (7)

To model Huntington's disease in OTCs, a broad array of established methods is available. (22) First, disease properties can be mimicked with OTCs from transgenic mice (37) expressing a part of the human huntingtin gene. (38) Second, the injection of neurotoxic substances like kainic acid, quinolinic acid or 3-nitroproprionic acid provokes selective γ-aminobutyric acidergic (GABAergic) neuronal death typical for the condition in the striatum. (22) Third, slices were successfully genetically modified through genetic transfection with the human pathological huntingtin gene. (39) Fourth, a group developed a model of Huntington's disease, in which all brain areas of interest are present in a single slice, and which manages to induce GABAergic neuronal death only by slicing inherent to the OTC method with coronal slices of postnatal rats. (22)

With a variety of methods, OTCs have been adapted to model traumatic brain injury. They include the weight-drop model, in which a weight is released to fall onto a specific region of the slice, the tissue stretch model, where a controlled tissue strain is applied, or involve rolling a steel cylinder over the tissue. (40–42)

Ischemic stroke has been modelled in organotypic slice cultures by oxygen-glucose deprivation. Hippocampal slices were used to elucidate processes such as energy depletion, excitotoxicity, mitochondrial impairment, or oxidative stress, all of which lead to neuronal cell death. (41)

OTCs were used in disease modelling for infectious diseases as well. In such a model, OTC preparations infected by tick-borne encephalitis virus were shown to be suited for the study of the disease caused by it. (43) What is more, small interfering RNA was proven in-vitro in an OTC-based model to be a potential antiviral treatment worth for further investigation in in-vivo models. (44)
OTCs have also been employed in prion research, for example using postnatal mouse cerebellar tissue. (45)

1.4 Assessment of viability in brain slice cultures and viability in neonatal vs. adult brain organotypic cultures

Cell death is a critical problem to consider when using OTCs for research, especially the survival of neurons is challenging. It has to be taken into account that in OTCs, there will be a substantial amount of axonal damage, (compare to this section (6)) which typically leads to neuronal death. (46) Humpel highlights the importance of factors like age of the donor animal, culture medium formulation, thinning of slices during the culture period, the speed at which the tissue block is cut into slices, sterility of the environment during slice preparation, health of tissue donor animals, etc. for neuronal survival in organotypic slices. (6)

But how should viability be assessed? Humpel, who mostly worked with postnatal brain tissue from mice and the membrane-interface technique, claims that a grey colour, thinning and attachment of slices to the membrane during culture are macroscopic signs of tissue survival, of which thinning is the most substantial. The slices would start to adhere to the membrane surface as early as a few days after the tissue has been placed in-vitro and this process ought to be completed after two weeks. In addition, the tissue should become transparent and flatter. (6) It was reported that membrane interface OTCs thinned down from initially 400µm to about 150/100µm. (14)

To gain a more accurate understanding of cell viability within a tissue slice, viability can also be objectified by using both living and fixed tissue. In living tissue, staining with fluorescent agents combined with confocal laser scanning microscopy is a widely used technique. Fluorescent staining probes capable of detecting cell death work through a broad variety of mechanisms: There are nuclear stainings marking the nucleus of the cell. Among those nuclear stains are, for the one thing, impermeant dyes like propidium iodide, ethidium homodimer-1 and -2 (EthD-1/2) and ethidium bromide, which are all suitable for detection of later phases of cell death and are applicable in live tissue, which can be stained multiple times over the course of the cultivation in-vitro. These molecules are expelled from healthy cells by active transport. In disrupted or dying cells, however, they permeate the membrane and are therefore suitable for the detection of dying and dead cells, regardless of whether necrosis or apoptosis is the underlying cause of cell death. For the other thing, there are permeant dyes, that are capable of plasma membrane penetration (such as Hoechst 33342, acridine orange and YO-PRO-1 iodide). Other types of

fluorescent cell stains include cytoplasmatic, mitochondrial or membrane stains, which stain the respective cell compartment. Calcein acetoxymethyl ester (calcein-AM), for example, is a molecule applicable as a cytoplasmatic fluorescent stain, since its ester groups are broken down by intracellular esterases in live cells and it is thereby converted to calcein, which shows green fluorescence. (47) Of note, it can also be used as a mitochondrial marker of apoptosis, as it is incorporated into the mitochondrial matrix by mitochondrial permeability transition pore protein, which is involved in apoptosis. It needs to be considered that fluorescent dyes employed for studies of cell death were originally developed for cells dispersed in a liquid for flow cytometry. As a result, issues such as poor tissue penetration and high background signals can occur in tissue slices. (compare to this section (6,25))

Another technique, that allows for viability assessment in living tissue slices is measurement of fluorescence emission. This method also utilizes fluorescent stains, for instance propidium iodide. (40)

Viability in slice cultures can also be evaluated with metabolic assays such as the lactate dehydrogenase (LDH) activity assay, (48) which is a colorimetric assay, that quantifies the amount of lactate dehydrogenase, an intracellular enzyme which can serve as a marker for cell damage, apoptosis, and necrosis, as it is released into the cell culture medium after cell death. Lactate dehydrogenase catalyses first, the conversion of the substrate lactic acid to pyruvate, and second, the build-up of reduced nicotinamide adenine dinucleotide (NADH) as a by-product. NADH, in turn, reacts with a tetrazolium salt - of which there are several (49) – resulting in a colour change. The absorbance of this solution is directly proportional to the quantity of LDH, which at the same time is directly proportional to the number of cells with impaired membrane function, or the number of dead cells respectively. (compare to this section (50))

Some studies have employed yet another possibility to assess viability and function of neurons in live brain slice cultures. They applied electrophysiological methods, for instance, the measurement the population spike amplitude, (51) a marker that Andersen and colleagues associated with the number of electrically active neurons. (51) Other electrophysiological methods include the measurement of spontaneous electric activity or of evoked potentials. They are of importance, since some other methods mentioned above

for assessment of viability utilize less specific fluorescent markers, which can only provide information about viability of all cell types, but not specifically about neurons. (6)

Yet, if one wishes to evaluate viability for a certain cell type such as neurons or astrocytes, immunohistochemistry can provide this information. Therefore, fixed tissue needs to be stained with, for example, apoptotic markers as well as cell-specific markers such as microtubule-associated protein-2, which is a neuronal marker, glial fibrillary acidic protein to label astrocytes, laminin (vessels) or CD11b (microglia). (compare to this section (6))

Taken together, what statements have we been able to make about viability in OTCs in general, or in postnatal and adult slice cultures more specifically? Generally, it is accepted, that embryonic tissue survives well in culture. (6) There are many published studies that applied postnatal OTCs to their specific research questions indicating sufficient survival of postnatal tissue in OTCs for a variety of studies. In contrast, the widespread application of adult OTCs (excluding acute slices mostly used in electrophysiological experiments) was not seen so far, which suggests ongoing challenges with this method. (26) A study investigating viability in postnatal and adult hippocampal slices from mice, found that from day 3 in-vitro onwards postnatal slices retain relatively stable viability or only a mild decrease in viability over time. Adult tissue, though, was affected by continuous cell damage during the culture period and finally resulted in degradation of the tissue slice. The results from this study were also indicative of neuronal cell loss specifically, detected through electrophysiological and morphological investigations. The authors claimed that the cell loss in adult OTCs was progressive and directly correlated with time in culture as well as it was consistently repeatable on further occasions. Upon treatment of adult slices with the growth-promoting and neuroprotective molecule BDNF (brain-derived neurotrophic factor), they observed better viability and delayed cell death. Owing to these results, they proposed the use of adult hippocampal OTCs as a model of neurodegeneration suitable for research on neuroprotection. (compare to this section (26)) Another investigation studied adult rat hippocampal slice cultures and these results were somewhat in line with those of the previously mentioned study. Specifically, they reported increasing trypan blue staining (a marker of cell damage and death) during culture, loosening of neuronal layers and slice thinning between days 6 and 10 in culture. In addition, based on caspase-3 detection by immunohistochemistry, they found that apoptosis did not occur immediately but only a few hours after slicing the brain tissue and ended at around day 6,

whereas cell degeneration went on, which suggests ongoing necrosis especially after the first few days in culture. (compare to this section (52))

With regards to these difficulties in culturing adult OTCs, some authors state that there is indeed a substantial need for adult OTCs. (53,54) This is, because even though there are a number of studies modelling adult pathologies in postnatal tissue, for instance Alzheimer's disease (55) or traumatic brain injury (56), postnatal OTCs differ from adult tissue in-vivo or adult OTCs in a number of characteristics: First, the older the animal, the faster the synaptic maturation in-vitro. (57,58) Second, postnatal slices show anomalous synaptic activity possibly due to the development of some neuronal pathways (i.e. the mossy fibre pathway) in-vitro. (58,59) Third, the distribution of certain glutamatergic receptors in postnatal hippocampal slice culture tissue, which had developed in-vitro, was revealed to be different from the distribution of this receptor in tissue of the same age matured in-vivo. (58,60) Finley and colleagues directed the attention to these differences between postnatal and adult brain tissue and to the according differences in gene expression, which might lead to misinterpretation with regards to the role of these genes in the adult brain. (58) All these findings suggest that postnatal tissue, which develops in-vitro, is different from adult brain tissue and might therefore not be suitable for the investigation of adult brain (patho)physiology. (compare to this section (58))

With regards to this necessity, which efforts have been successful so far in establishing adult brain OTCs? Based on viability assessment by population spike measurement, one study found that it was feasible to culture adult rat hippocampal slices until day 6 in-vitro if the culture conditions were suitable. This study optimized a medium, whose composition was similar to cerebrospinal fluid. (51,53) It was reported that hippocampal slices from young adult rats (P20-21) cultured for 7 days preserved hippocampal anatomy, which resembled adult hippocampus more than postnatal slice hippocampus. Furthermore, synapses and hippocampal circuitry remained functional. (53,58) Another group gave an account of high cell survival in hippocampal OTCs from mice as long as 30 days in-vitro when treated with serum-free medium. The number of cells stained with DAPI (4′,6-diamidino-2-phenylindole) in slices treated with serum, though, continuously decreased from the beginning of the culture. (53,54) These results are suggestive of a promising room for improvement of the survival of adult brain OTCs through modifications in culture conditions.

1.5 Culture media

Generally, culture media for mammalian cells consist of cell culture grade water void of bacterial or endotoxic contamination, ions (sodium, potassium, calcium, magnesium, phosphate, chloride, bicarbonate adjusted to osmolality and pH suitable for the cell type), an energy source like glucose or glutamine, amino acids, vitamins like folic and pantothenic acid, niacinamide, thiamine, riboflavin, pyridoxal as well as inositol and choline. In classical media, a protein source like animal serum is typically included in the formulation, which contains a variety of proteins, hormones, growth factors, lipids, trace elements etc. However, the advantages of serum (overall good cell growth) come at the expense of potential cell overgrowth, contamination with microbial organisms or prions, antigenicity of protein residues such as antibodies able to reduce viral replication in-vitro, induction of cell differentiation in stem cells, issues with purification of cell products or experimental inconsistency. The latter is often due to high variability between different serum lots from the same company since serum composition is influenced by the health and diet of the donor animal and even by harvest time. If therefore a serum-free medium is desired, substances like zinc, manganese, selenium, copper, insulin, fatty acids etc. can be added to substitute for serum components.

The exact composition of cell culture media usually depends on the cell (or tissue) type (including if the cells attach to a surface or need to be cultured in a cell suspension) and the research purpose. The classical media were originally designed for tumour cell lines and are not appropriate for stem cells or differentiated cells such as neurons or liver cells. These cell types require more adaptation to piece together a suitable medium. (compare to this section (61))

Media are easy to handle and to adjust to the needs of the tissue. If a certain cell culture medium or supplement was able to significantly improve cell survival in adult OTCs, it would make complicated culture set-ups, which can, for instance, include perfusion pumps, (62) obsolete.

1.5.1 Promising medium formulations and supplements for adult organotypic brain cultures

1.5.1.1 Serum-free media

Serum, which is obtained from animals, is a somewhat controversial additive to cell culture media. Serum promotes cell growth but has disadvantages (see chapter 1.5). In general, many cell culture medium formulations used for brain OTCs include around 20% horse

serum. (24,54,63) Serum deprivation is known and even used to induce neuronal death. (25) However, a group has claimed to have successfully maintained adult OTCs in culture, with a few neurons surviving up to day 30 in-vitro. In this study, slices treated with serum (short-term and continuously) performed worse than their serum-free counterparts. All the media in this study were based on Neurobasal A with B27 supplement. (54) Nevertheless, there are only few reports that I am aware of further investigating the effect of serum on organotypic brain slice cultures. Consequently, whether a serum-free medium based on Neurobasal A, which is designed for neuronal tissue, (64) proves to be beneficial for overall adult OTC viability in comparison with serum-containing medium is a question worth confirming. Furthermore, the effect of serum on brain regions other than hippocampus and when combined with other additives might be an interesting research area with potential impact on cell survival.

1.5.1.2 Insulin

Insulin functions as a growth promoting, endogenous molecule with a variety of beneficial effects on central nervous system tissue. (65) It is already used as a growth-promoting additive in cell culture media (66) and is also found in serum, (67) which is why it also occurs in serum-enriched classical medium formulations.

Insulin is shown to play a role in developing nervous tissue. Insulin causes cells to incorporate thymidine and uridine, which are components of nucleic acids. (68) In glial cells, insulin enhances protein synthesis (69) and in neuronal cells the insulin receptor also plays a role in growth. (70) In addition, insulin and Insulin-like growth factor 2 were found to be essential in NGF (nerve growth factor)-mediated axonal growth in human neuroblastoma cells (71) and additionally require astrocytes. (72) Insulin was claimed to promote growth in astrocytes stemming from rats (73) and humans. (74) Insulin also enhances the expression of a postsynaptic structural protein in rat hippocampus. (75) It regulates the development of dendrites and excitatory synapses in hippocampal neurons. (76) Insulin causes multipotent neuronal stem cells to undergo proliferation and differentiation, lack of insulin is responsible for their cell death by autophagy. (77) Furthermore, insulin is shown to have neuroprotective properties protecting against apoptosis, ischemia as well as beta-amyloid toxicity and it is known to neutralize central nervous system oxidative stress. (compare to this entire section (65)) For all these reasons, and especially for its neuroprotective effect acting against ischemia, apoptosis and

oxidative stress, insulin might support overall cell survival in adult rat brain slice cultures, which is why insulin was chosen to be one of the substances investigated in this study.

1.5.1.3 Ascorbic acid

Oxidative stress commonly leads to apoptosis and necrosis (78,79) and neurons are especially vulnerable to it because of their content in lipids, and their highly active metabolism. (79) Ascorbic acid (AA, also called vitamin C) is known to be a free radical scavenging substance, and thus capable of reducing oxidative stress. (79) This is why vitamin C is considered to be exceptionally important for healthy brain function and it also plays a role as a co-factor in biosynthesis of amino acids and proteins. (80) Deficiency of ascorbic acid has been linked with numerous neurological (for instance Alzheimer's disease) or psychiatric disorders (like schizophrenia). (79) In a study aiming to improve cell viability of adult rat hippocampal organotypic cultures, ascorbic acid was found to slightly improve neuronal viability especially when combined with insulin. (51) Another group recently published a report about the positive effect of long-term AA supplementation on cell survival in aged hippocampal OTCs from rats. (79) For these reasons, the effect of AA on adult rat brain slice cultures (including not only hippocampus, but also thalamus and cortex) was investigated in this study.

1.5.1.4 Growth factors

Humpel, who regularly works with organotypic cultures of mice, highly recommends the use of growth factors with OTCs and even states that they are critical to ensure the viability of vulnerable neurons in OTCs, such as cholinergic neurons, which require NGF. (6) BDNF is shown to have powerful survival-promoting effects in hippocampal OTCs from adult mice. (26) BDNF, for example, could be an interesting additive for cell culture media of adult OTCs as it has positive effects on both growth and survival of several different neuron populations. (81) Of note, Humpel claimed to have had no success in prolonging the viability of adult OTCs by the use of growth factors. (6) Unfortunately however, he did not present data on this topic. As this study aims to identify a substance, which promotes overall cell survival as opposed to survival of a single cell type, growth factors were not investigated in this work.

1.6 Unanswered questions

Unfortunately, there is only little data on the effect of certain cell culture medium components or additives on overall cell survival in adult rodent OTCs. Kim et al. showed that a serum-free alternative can be used with adult hippocampal brain slices from mice and might be able to sustain adult neurons. (54) However, this study did not compare their Neurobasal A-based media with other commercially available, more classical medium formulations. These traditional formulations typically include serum and are often based on Basal Medium Eagle (BME) or a similar medium. (82–84) Thus, it remains enigmatic whether a Neurobasal A-based, serum-free medium is superior to a BME-based, serum-enhanced medium. Another study found clues that the combination of insulin and ascorbic acid might be favourable for the survival of hippocampal OTCs from adult rats. (51) However, to my knowledge, there are hardly any studies investigating insulin, AA or their combination in comparison to media without them over a period of time in adult OTC, leaving their effect on adult OTC elusive. Further, whether a medium composition could support viability in a whole-brain slice that encompasses multiple brain regions like cortex, hippocampus or thalamus, is of critical interest in order to turn organotypic slice cultures into a useful tool to study, for instance, neuronal connectivity in ex-vivo conditions.

1.7 Aims and hypotheses

The ultimate aim of the present study is to improve in-vitro viability of OTCs of coronal, whole-brain slices from adult rats, which could potentially ensure an adequate time frame for further investigations, like pharmacological or neuromodulatory interventions and assessment of molecular mechanisms. On that quest, the determination of a reliable temporal profile of in-vitro viability of these cultures and detection of possible brain region-dependent differences are essential and this comprises the second goal of the study.

The main hypothesis of this work is that the serum-free medium formulation with Neurobasal A (NB-A) increases viability in coronal slice cultures of adult rat brain in whole-brain, cortical, hippocampal and thalamic regions when compared to a traditional serum-containing medium based on Basal Medium Eagle (BME). Further, the second hypothesis is that vitamin C and/or insulin supplementation has a positive effect on these brain tissues.

These hypotheses were tested in an experimental set up, where coronal brain slices from adult animals were assigned for cultivation over a 7-day period to one of the following control and experimental groups:

1. BME + 25% HS (horse serum)
2. BME + 25% HS + insulin
3. BME + 25% HS + vitamin C
4. BME + 25% HS + insulin + vitamin C
5. NB-A
6. NB-A + insulin
7. NB-A + vitamin C
8. NB-A + insulin + vitamin C
9. dead control

Viability of the cultures at 1, 3, 5 and 7 days in vitro was assessed with LIVE/DEAD staining (see chapter 2.1), where live cells were marked with calcein-AM and non-viable cells with EthD-1. (85) Images obtained using a confocal laser scanning microscope were then analysed with the help of an image processing software to calculate the number of live cells as a percentage of whole cell counts (live and dead).

2 Materials and Methods

2.1 Materials

For tissue harvest and preparation of the organotypic culture:

Devices: Vibratome Leica VT 1200S, microwave oven, water bath, sterile cell culture bench.

Instruments: Scissors, scalpel, vibratome blade, brushes for slice transfer, embedding dishes for preparation of agarose blocks, micropipettes.

Consumable goods: syringes and injection needles, petri dishes, membrane inserts (MF-Millipore, pore size 0.45µm, diameter 3 cm, Merck, Germany), Falcon tube (50 ml), six-well plates, pipette and micropipette tips, phosphate buffered-saline (PBS), pentobarbital sodium (Exagon 400mg/mL, Richter Pharma, Austria), culture media, agarose powder, glucose (30%, diluted in PBS), superglue.

Composition of cell culture media:

For 100 mL of BME-based medium: BME (Gibco, Thermo Fisher Scientific, MA, USA) 50 ml, Hank's Balanced Salt Solution (HBSS, Gibco, Thermo Fisher Scientific, MA, USA) 25 ml, heat-inactivated horse serum 25 ml (Gibco, Thermo

Fisher Scientific, MA, USA), GlutaMAX 500 µl (Gibco, Thermo Fisher Scientific, MA, USA), 30% glucose solution 1 ml, 100µL Normocin (50mg/mL, Invivogen, CA, USA) and/or 500µL of Penicillin/Streptomycin (10.000 U/mL, Gibco, Thermo Fisher Scientific, MA, USA). Insulin supplementation was done with addition of 100 µl of a 10 mg/ml stock solution of human recombinant insulin (final concentration 10mg/L, Roche, Switzerland). Ascorbic acid supplementation was done with addition of 5mL of a 10mM stock solution per 100mL medium (final concentration 500µmol/L, Thermo Fisher Scientific, MA, USA).

For 100mL of Neurobasal-based medium: 97mL Neurobasal A (Gibco, Thermo Fisher Scientific, MA, USA), 2mL B27 serum-free supplement (2%, Gibco, Thermo Fisher Scientific, MA, USA), 1mL GlutaMAX (Gibco, Thermo Fisher Scientific, MA, USA), supplement of 100µL Normocin (50mg/mL, Invivogen, CA, USA) and/or 500µL of Penicillin/Streptomycin (10.000 U/mL, Gibco, Thermo Fisher Scientific, MA, USA). Insulin and/or ascorbic acid supplementation was performed in the same way as in the BME-based media.

Staining:

Dulbecco's Phosphate Buffered Saline (D-PBS, Gibco, Thermo Fisher Scientific, MA, USA), LIVE/DEAD Viability/Cytotoxicity Kit for mammalian cells (Thermo Fisher Scientific, MA, USA) including calcein-AM and EthD-1 working solutions as delivered by the manufacturer, 0.5 ml of each ethanol (70%) and Dimethyl sulfoxide (DMSO, 100%, Sigma-Aldrich, MO, USA) for killing a slice to use it as a dead control, a 15 ml falcon, aluminium foil for light protection, several pipette tips and disposable cell culture pipettes.

For imaging and subsequent fixation of slices for storage:

LSM 510 confocal fluorescence microscope with ZEN 2007 software, a thin glass bottom Petri dish, a brush for transfer of slices, 4% formaldehyde, Parafilm.

Animals:

9–12-week-old adult, male Sprague-Dawley rats. They were taken care of at the Institute of Biomedical Research at the Medical University of Graz. Water and food provided ad libitum.

Microbiological smear tests:

Cotton swabs (sterilised), petri dishes with solid, non-selective growth medium for bacteria/fungi.

2.2 Methods

2.2.1 Tissue harvest

The animals were shortly sedated in an anaesthesia chamber using isoflurane in order to ensure stress-free intraperitoneal injection of sodium pentobarbital (2mL/kg body weight). Upon reaching deep anaesthesia (confirmed with asystole), the head was removed right below atlas with a large pair of scissors, briefly immersed in an ice-cold 70% ethanol solution, again washed in ice-cold sterile PBS. The skull was then opened with the help of a small pair of scissors. The brain was gently removed with caution to prevent tissue trauma, placed in a falcon tube containing an ice-cold PBS solution containing a final concentration of 66.6mmol/L glucose and transferred to a bench with laminar airflow suited for cell culture applications.

2.2.2 Slice preparation and cultivation

The brain stem and cerebellum were cut off with a sharp blade, the cerebrum was then placed in a metal dish cooled on ice. Warm, liquified agarose (2.5%, dissolved in MEM) was poured over it until fully covered. Once solid, the agarose block was then glued to the stage of the vibratome, immersed in ice-cold PBS with glucose (30%) and slowly cut with the vibrating blade at an amplitude of 1.5 mm in 200µm thick slices. With the help of a rat brain atlas, it was ensured macroscopically, that all slices chosen for culture contained the cortical, hippocampal, and thalamic brain region. (86,87) With a brush, slices were carefully transferred onto a single membrane insert in a six-well plate, that had already been carefully placed onto pre-warmed cell culture medium without trapping air bubbles underneath the membrane. Samples were transferred to the incubator as fast as possible, where they were kept at culture conditions (37°C, 5% CO2).

The media were refreshed every two days or earlier in case a change in colour occurred indicating an acidic pH.

To test for bacterial or fungal infection, sterilised cotton swabs were used for smear sampling from the surface of slices treated with NB-A + vitamin C. The specimen was then transferred onto the surface of the petri dish coated with non-selective solid growth medium for bacteria/fungi. Subsequently, the covered petri dish was stored upside down in an incubator at 37°C for a week. There was no bacterial or fungal growth even after a week of sampling.

2.2.3 Staining

On days 1, 3, 5 and 7, six slices of each treatment condition were stained using the LIVE/DEAD Viability/Cytotoxicity Kit for mammalian cells (Thermo Fisher Scientific, MA, USA), which is accessible in retail sale and that contains calcein-AM (staining the somata of living cells in green) and EthD-1 (marking the nuclei of dead cells in red). An additional slice for each condition was incubated with 1:1 DMSO (100%) and ethanol (70%) for ten minutes prior to staining, which served as a dead control. Of note, there were no repeated measurements on the same slices. On each staining day, different slices were used.

The slices were incubated for 30-45 minutes at room temperature according to the manufacturer's instructions and washed with D-PBS.

2.2.4 Microscopy

The stained tissue was then placed on a petri dish with a thin glass base for imaging at the confocal laser-scanning microscope with two lasers (excitation wavelength of 488nm and 543nm (approximate excitation/emission 495nm/515nm for calcein and 495/635nm for EthD-1 according to the manual provided by the manufacturer). (85) Images of the tissue containing the entire signal emitted by cells (10x magnification, approximately 5x5 tiles depending on tissue size, z-stack with 5 µm between each plane, pinhole 1 aery unit, laser intensity adjusted to tissue fluorescence in both lasers) were taken and saved.

2.2.5 Slice exclusion

Starting from day 3 in-vitro and increasingly over time, the extensive growth of unknown, round to oval cells, which were strongly labelled by calcein but never by ethidum-homodimer-1 (except for dead control slices), occurred in all groups. They were most frequent on the surface of the slices and seemed to be most abundant on hippocampal tissue. Initially, their origin (microbial contamination vs. proliferation of brain cells) was unclear. Microbiological analysis (see chapter 2.2.2) of slices cultivated in Neurobasal A + vitamin C did not reveal bacterial or fungal growth even after a week of sampling, even though the growth of unknown cells was confirmed on these slices by microscopy. Therefore, the most likely explanation was that they are astrocytes forming a carpet, or glial scar. Although in vitro formed glial scar tissue is a natural part of the cultured organotypic slice, (25) images, on which extensive numbers of them covered a large (~70% by qualitative assessment) area of the culture, were excluded from the analysis for the following reasons:

1. Their abundance was irregular across the brain regions (most abundant on hippocampal region) and would have otherwise led to a region-specific bias in viability assessments.
2. They were sometimes washed off the slice surface into the surrounding liquid at the washing steps during preparation for laser scanning microscopy, which would have contributed to a bias by the addition of disproportionate variability.

2.2.6 Data analysis and quantification

A random number was assigned to each image and the image files were renamed with their respective number before analysis for blinding and bias prevention during quantification.

The number of cells labelled as dead and alive was assessed automatically by the BoneJ cell counting plug-in (version 7.0.7) (88) of an image processing software (Fiji Is Just ImageJ, version 1.53c). (89)

First, each .lsm-file was split in its two channels (red for EthD-1 staining, green for calcein) the command “Find Edges” was used in order to discover cells, then they were converted to an 8-bit greyscale image for further image processing. Subsequently, the image was converted to a binary image using the “Triangle” algorithm (90) for both channels and afterwards the commands “Watershed” followed by “Fill Holes” were carried out to optimize the segmentation result. Finally, the object counter function of BoneJ plug-in was used, the threshold set to 128 and the size filter set to 40-max. for ethidium-1-homodimer staining and 41-max. for calcein staining. A maximum size limit was not established, as larger, confluent objects were well separated by the “Watershed” operation. In addition, large objects, which did not represent cells, were very rare and a number of upper size limits did not alter cell count results in any significant way. The live and dead cells were counted in three fields of view (FOV) per slice in each region of interest (cortex, hippocampus, thalamus) and the three live cell counts as well as the three dead cell counts from the same slice were summed up to model whole-slice viability (referred to as “total”) as the entire brain slice was too large to only take one image of. Finally, viability was expressed as a percentage of living cells in all cells (live and dead). This was done for cortical, hippocampal, thalamic and total cell counts resulting in four viability values per slice.

The data was summarized and presented in box-whisker plots, where 2nd and 3rd quartiles are depicted as boxes, 1st and 4th quartiles as whiskers, and median values as a bar dividing 2nd and 3rd quartiles. Across the text, viability results are provided as mean ± standard error mean (SEM) unless indicated otherwise.

2.2.7 Statistical analysis

Statistical analyses were performed using SPSS (version 28.0.1.0). (91) For each experimental day (1, 3, 5, 7), the respective cell count results (cortex, hippocampus, thalamus and total) were compared to 1) the medium control

(BME medium without supplements), 2) the pooled dead controls from all groups of the same day and 3) to each other.

Normality of the data distribution was evaluated by a combination of both visual interpretation of Q-Q-plots and Shapiro-Wilk test for each group. Variance homogeneity was tested by Levene's test. Statistical comparison between the groups that have a normal distribution was done with one-way analysis of variance (ANOVA) followed by Gabriel's post-hoc test, as sample size between groups was different and Gabriel's test is intended for this situation. (92) When a significant heterogeneity was observed, Welch's ANOVA was used, and pair-wise comparisons were performed with Games-Howell's post-hoc test.

For comparisons, where one or more of the experimental groups showed non-normal distribution or had such a low sample size ($n<3$), that it did not allow a reliable normality check, non-parametric tests were used. These comparisons were conducted using Kruskal-Wallis H Test followed by pairwise analyses with Bonferroni correction.
In any statistical test, a p-value < 0.05 was deemed statistically significant.

To illustrate statistically significant differences between the treatment groups, black asterisks (* if $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) and significance bars were utilized in the figures of the results section. Likewise, red asterisks were used in these figures to highlight a statistically significant difference from dead control. In addition, all occurring significances between any two groups were always reported within the corresponding text in the results section. Please refer to
Table 1 - Table 11 in the appendix for more detailed lists of the statistical tests used per group and the respective results.

3 Results

3.1 Total cell counts

3.1.1 Day 1

At day 1 in-vitro, average viability of whole slices (total) combining the cell counts of cortex, hippocampus and thalamus that were cultivated with serum-containing BME was approximately 50 percent (n=7, 47.68% ± 9.03), although it was as high as about 80% in some of the slices (Figure 2Figure 2: Day 1 (total), viability by medium.). While supplementation of insulin led to a slight reduction in tissue viability, vitamin C-supplemented slices showed a better preservation, where live cells comprised ≥40% of all cells in nearly all slices. Strong reductions of viability were observed, when serum-containing BME was supplemented with both insulin and vitamin C, indicating that a combination of both was not well tolerated at least in the first day of culture. It should be noted, however, that these differences were not statistically significant.

Viability in the serum-free NB-A group was comparable to serum-containing BME group (n=6, 47.4% ± 6.60). While insulin supplementation resulted in a slight improvement of viability, a reduction was observed in vitamin C-supplemented slices, although these differences were not statistically significant. These observations are noteworthy as insulin and vitamin C supplementation influenced slice viability differentially, yet the effects were in the opposite direction in the NB-A group compared to the BME group. When serum-free NB-A media were supplemented with both insulin and vitamin C, however, there was no detectable influence on the slice viability, which contrasts with the unfavourable effect of the same combination in the serum-containing BME group. Amongst all treatment groups, the only statistically significant difference was detected between insulin-supplemented serum-free NB-A and serum-containing BME supplemented with both insulin and vitamin C ($p=0.024$).

It should be noted that all experimental groups showed statistically significant differences from the dead control ($p=0.000$-0.026) except the group with serum-containing BME + vitamin C.

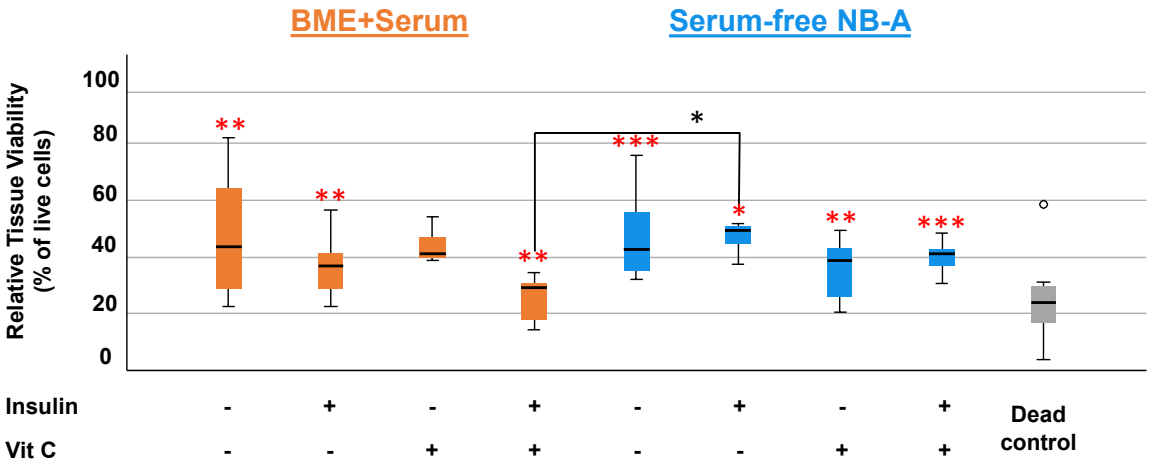

Figure 2: Day 1 (total), viability by medium.
Additionally, all groups were more viable than dead control in a statistically significant way.

3.1.2 Day 3

On day 3 in total cell counts, the slices treated with serum-containing BME showed a mean viability of 20.26% ± 7.20 (n=7; Figure 3). Notably, some single slices of this group had a viability of more than 50%, pointing towards a high variability similar to day 1 samples. The addition of insulin increased mean viability only marginally (22.72% ± 6.86, n=7). The supplementation of vitamin C in the BME-based medium, however, seemed to be beneficial on this day as it increased viability much further (35.18% ± 6.51, n=4) and this effect was also observed in the BME + vitamin C + insulin group (39.1% ± 6.46, n=6). These differences did not reach statistical significance.

Viability in slices cultured in serum-free NB-A medium was significantly higher (48.40% ± 2.27, n=6; one-way ANOVA: $F(6, 32)=2.81$, $p=0.026$; Gabriel's test: $p=0.043$) compared to those cultured with serum-containing BME. It should be noted that these viability levels were comparable to those observed at day 1, indicating a successful preservation of tissues with serum-free NB-A, in contrast to the substantial cell loss in the serum-containing BME-group. Slices cultivated with serum-free NB-A supplemented with either insulin, vitamin C or both, had lower viability compared to those cultivated with serum-free NB-A alone (statistically not significant). Dead control group was significantly different from all the other groups ($H(8)=28.81$, asymptotic $p<0.01$; pairwise analysis Bonferroni correction;

15 comparisons: p=0.000-0.004) except for those cultivated with serum-containing BME alone (without insulin or vitamin C; referred to as medium control in the supplemented data sheets in the appendix), BME + insulin, and BME + vitamin C.

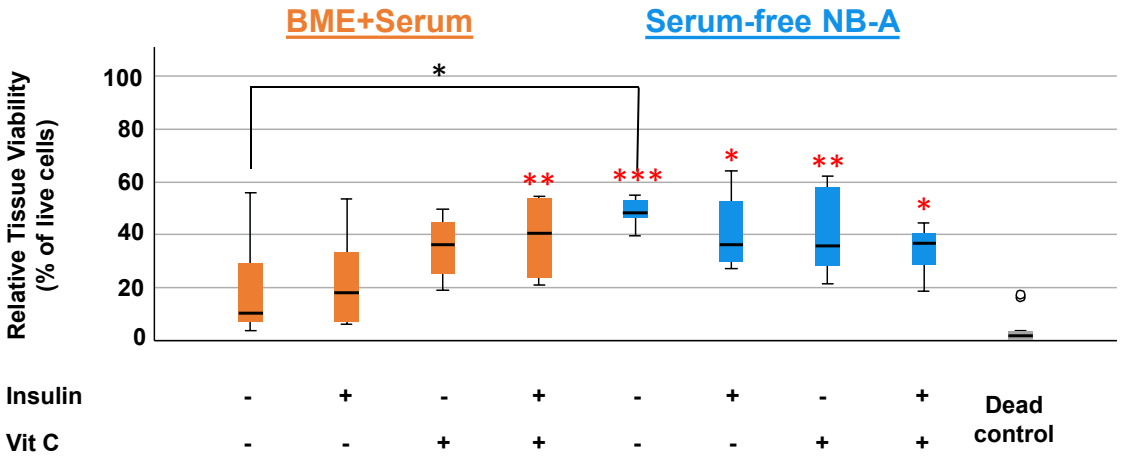

Figure 3: Day 3 (total), viability by medium.
Additionally, all groups were more viable than dead control in a statistically significant way except for BME, BME + insulin and BME + vitamin C.

3.1.3 Day 5

On day 5, viability of OTC treated with serum-containing BME was slightly higher than on day 3 (26.93% ± 8.79, n=6; Figure 4). Whilst those supplemented with insulin showed slightly higher viability (30.08% ± 6.02, n=6), those supplemented with vitamin C were less viable (19.95% ± 0.7, n=2, low sample size due to slice exclusion). Combined supplementation of insulin and vitamin C in BME did not provide better results and average viability on day 5 was reduced approximately by half compared to those at day 3 (24.25% ± 6.57, n=4). These differences were not statistically significant.

Slices cultivated with serum-free NB-A medium showed viability higher than those with serum-containing BME (not significant) and on a similar scale as BME with insulin (30.92% ± 5.21, n=5). The supplementation of insulin to serum-free NB-A did not greatly alter viability compared to NB-A alone (32.06% ± 4.93, n=4) and the vitamin C-supplemented group showed the lowest average viability amongst all serum-free NB-A groups (16.72% ± 1.55, n=4). Similar but less pronounced reductions were observed in those with combined supplementation of vitamin C

and insulin (23.34% ± 2.05, n=3). These reported differences were not statistically significant.

It should be noted, however, at day 5 none of the cultivation groups showed a statistically significant difference in viability compared to the dead controls, indicating the extent of degeneration and cell death reached to a very high level.

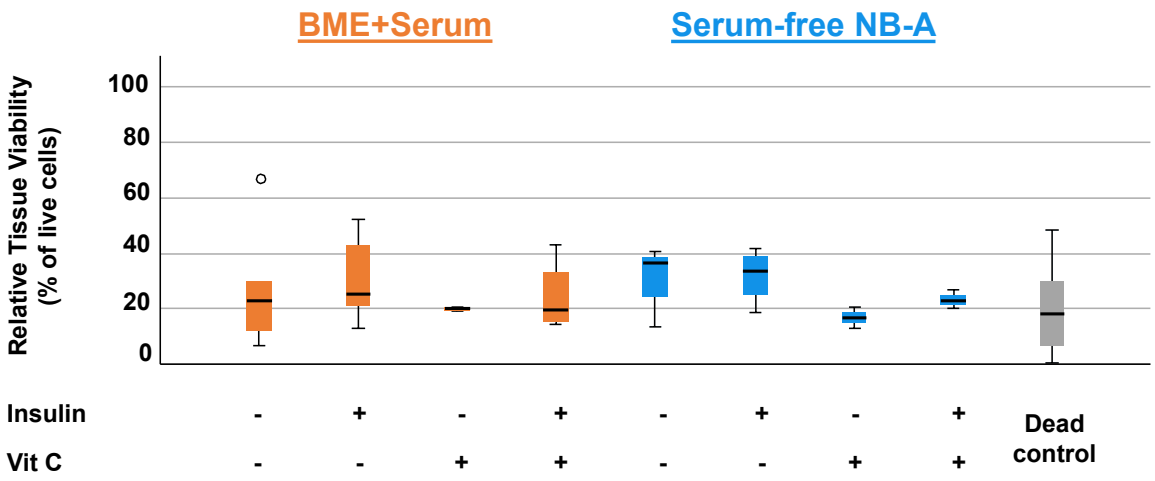

Figure 4: Day 5 (total), viability by medium.

3.1.4 Day 7

On day 7, sample sizes in several groups were relatively low due to slice exclusion and thus, statistical analysis was impaired. The highest mean viability of all groups was observed in the serum-containing BME group (41.48% ± 7.06, n=4). Viability in slices treated with BME and insulin was lower and reached a mean of 35.92% ± 3.03, n=4. Once again, the viability in OTC treated with BME and vitamin C was lower than that of BME alone (26.82%, n=1), which was also true for BME + vitamin C + insulin (29.25% ± 3.92, n=5). However, these differences were not statistically significant.

The observed viability in the serum-free NB-A group was clearly lower than that of slices cultivated with serum-containing medium (20.1% ± 8.09, n=2) and was similar when vitamin C or insulin/vitamin C were added (Figure 5). This is particularly noteworthy, as it contrasts with the observations with slices analysed at day 3 and 5, where serum-free NB-A consistently provided higher viability levels compared to serum-containing BME. These observed differences were not statistically significant.

Similar to day 5, the percentage of live cells in the slice cultures in any group was so low that a statistically significant difference was not observed when they were compared to the dead controls.

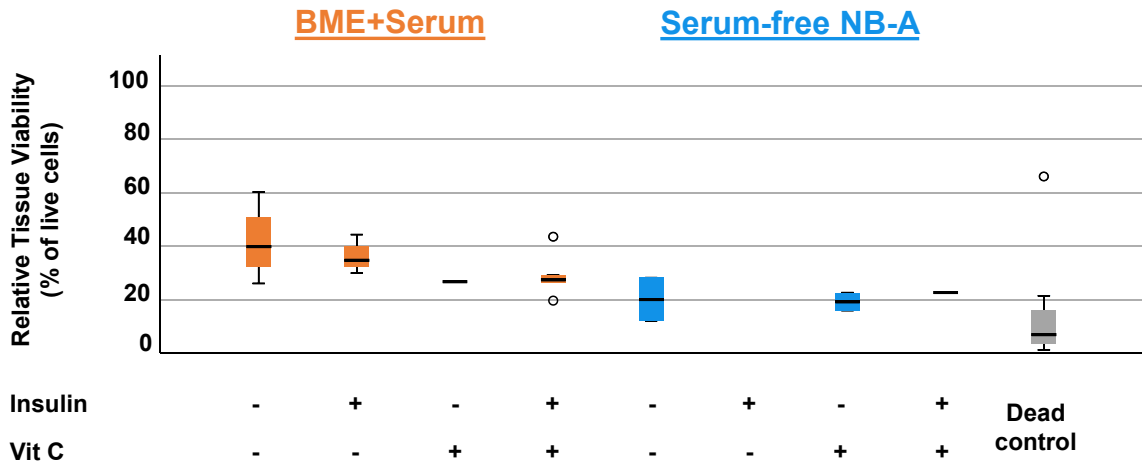

Figure 5: Day 7 (total), viability by medium.

3.1.5 Summary of total cell counts

In summary, total slice viability in serum-free NB-A was comparable to serum-containing BME on day 1 in-vitro, higher on days 3 and 5, but lower on day 7. The difference on day 3 was statistically significant.

Amongst the groups with BME-based media, additional insulin reduced viability slightly on day 1, increased it on days 3 and 5, and reduced it on day 7. As for ascorbic acid supplement, viability was similar, yet a little lower than for BME alone on day 1, increased tissue viability on day 3, and reduced it on days 5 and 7. The combined BME + vitamin C + insulin medium markedly decreased viability on day 1, and considerably increased it on day 3. On the remaining days in the second half of the experiment, this medium performed in similar fashion compared to BME + vitamin C. However, these observed differences were not statistically significant.

In NB-A, insulin supplementation did not consistently influence viability as it slightly increased viability on day 1, decreased it on day 3, while on day 5 no effect was visible. This pattern differed from the one observed in OTC treated with BME-based media. Vitamin C in NB-A reduced viability until day 5 and was comparable to NB-A alone on day 7. This pattern was also different in BME-based media, where ascorbic acid increased viability on day 3. Interestingly, a distinct effect of the combination of vitamin C and insulin in NB-A medium was not observable on days 1 (decrease to a similar extent as ascorbic acid), 5 (decreased viability but not

as strongly as ascorbic acid) and 7 (no effect observable). On day 3 however, it decreased viability to a greater extent than insulin or vitamin C supplement did on their own. These results, however, were not statistically significant.

3.2 Cortex

3.2.1 Day 1

After the first day in-vitro, mean viability of slices treated with BME without supplements came in at 50.3% ± 9.61 (n=6). In this tissue, cultivations in insulin- or vitamin C-supplemented BME showed reductions in the average viability (Figure 6, BME + insulin: 43.30% ± 7.08 (n=5); BME + vitamin C: 41.07% ± 2.88 (n=6)) along with a reduced variability and the median values quite comparable to that without supplementation. A remarkably lower tissue viability, on the other hand, was observed with serum-containing BME that had combined supplementation with both insulin and vitamin C (25.45% ± 4.44, n=6).

In the NB-A group, mean viability was in a similar range as in the BME group with 44.93% ± 7.89 (n=7). Insulin-supplemented NB-A group did not show much of a difference in average viability despite a smaller variance (42.39% ± 3.29, n=6) but lower viability levels were observed in tissue with vitamin C-supplemented NB-A (31.15% ± 6.58, n=6). When insulin and vitamin C were combined in NB-A medium, viability amounted to an average of 37.94% ± 3.48 (n=6), which was between NB-A + insulin group and NB-A + vitamin C group viability.

Observed differences were, however, not statistically significant.

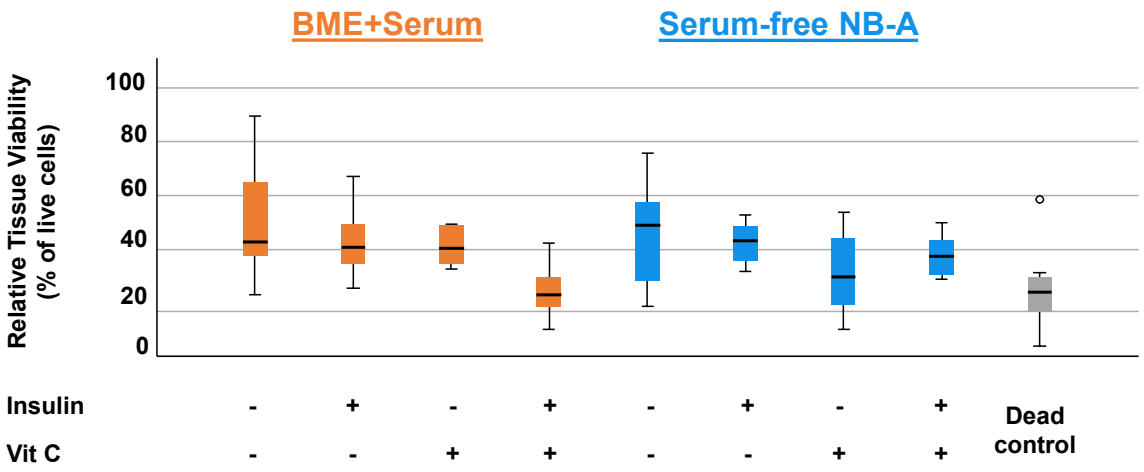

Figure 6: Cortex day 1, viability by medium.

3.2.2 Day 3

On day 3, cortical tissue cultured with BME media had very low mean viability (14.84% ± 5.82 SEM, n=8) compared to that on day 1. Cultures that were supplemented with insulin and especially vitamin C showed higher viability levels (BME + insulin: 25.21% ± 8.55 n=7; BME + vitamin C: 42.99% ± 10.40, n=3). The combination of insulin and vitamin C performed better than BME + insulin, but worse than BME + vitamin C (Figure 7). These differences, however, were not statistically significant.

Serum-free NB-A was significantly better than BME with or without supplements (65.45% ± 6.54 SEM, n=7). The statistically significant differences were detected compared to BME (p=0.008) as well as to BME with insulin (p=0.012). Notably, supplementation of insulin or vitamin C did not result in improved viability in the serum-free NB-A group, in contrast to serum-containing BME. Lower cortical viability was observed particularly in slices cultivated with NB-A containing insulin alone or in combination with vitamin C. NB-A supplemented with vitamin C resulted in better tissue viability compared to that of insulin supplementation, albeit lower than that with serum-free NB-A alone. These differences, however, were not statistically significant.

It should be noted that a statistically significant difference compared to dead controls was not observed in the analysed groups, except serum-free NB-A alone (p=0.000019) or with vitamin C supplementation (p=0.008).

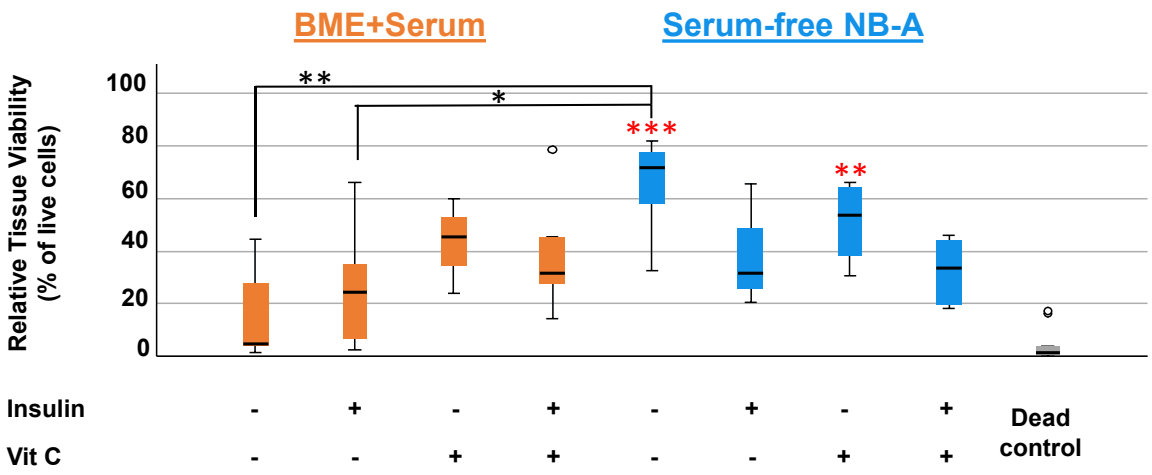

Figure 7: Cortex day 3, viability by medium.
Additionally, dead control differed from NB-A + vitamin C and NB-A in a statistically significant way.

3.2.3 Day 5

At day 5 in-vitro, mean viability amounted to 40.61% ± 11.64 (n=9) in the slices cultured with serum-containing BME, yet the observed variance was enormous (Figure 8). Supplementation of insulin, vitamin C or both to serum-containing BME did not result in statistically significant differences, although the average viability was lower and the variance was smaller (BME + insulin: 30.66% ± 7.09, n=7; BME + vitamin C: 30.24% ± 8.68, n=2; BME + vitamin C + insulin: 21.18% ± 7.70, n=2).

Slices cultured with serum-free NB-A medium with or without supplementation of insulin and/or vitamin C did not show significantly different viability levels compared to those observed in BME groups (NB-A: 30.25% ± 9.96 SEM, n=5; NB-A + insulin: 31.60% ± 6.6, n=4; NB-A + insulin + vitamin C 26.62% ± 5.28, n=3). Remarkably lower levels of average viability, however, were observed in slices cultured with NB-A supplemented with vitamin C (13.45% ± 2.68, n=4). In addition, dead control did not differ from any of the groups in a statistically significant manner.

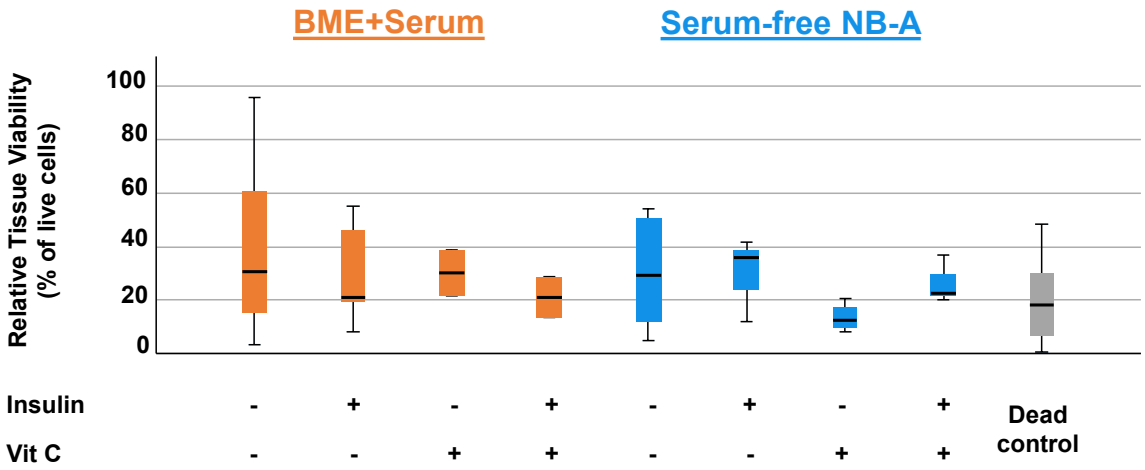

Figure 8: Cortex day 5, viability by medium.

3.2.4 Day 7

On day 7, slice exclusion due to the growth of unidentified cells resulted in very low sample sizes below three in most groups (compare Figure 9). Therefore, meaningful inferences could not be drawn from the data.

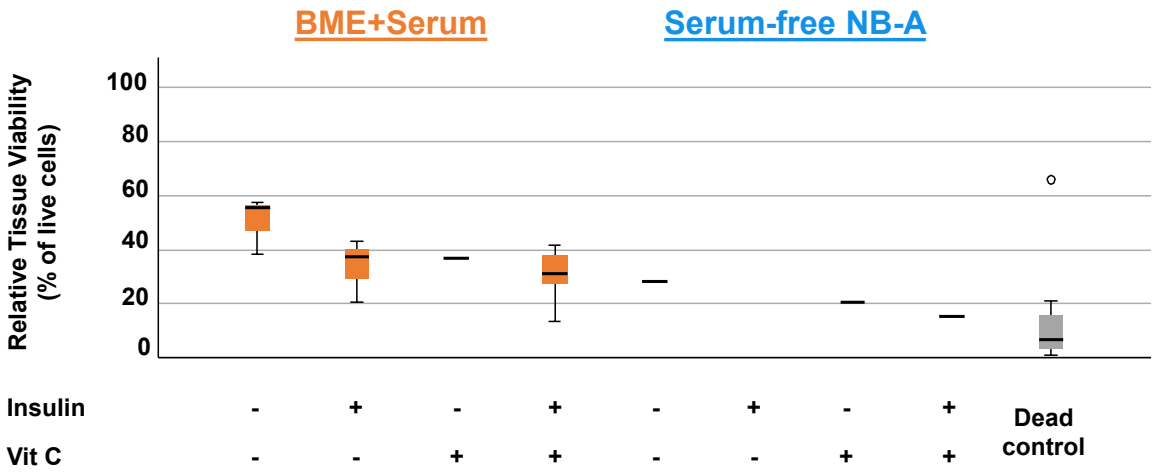

Figure 9: Cortex day 7, viability by medium.

3.2.5 Summary cortex

In cortical tissue (Figure 10), the comparison of serum-free NB-A and serum-containing BME (both without insulin or vitamin C supplement) showed that OTC cultivated with NB-A had significantly higher viability on day 3 ($p=0.008$). In contrast, viability in NB-A treated slices was lower on all other experimental days. However, these differences were statistically not significant.

In slices that were treated with one of the serum-containing media, a similar pattern was observed on days 1, 5 and 7 in-vitro: When supplement-free BME was compared to their counterparts with added insulin and/or vitamin C, both insulin and vitamin C supplementation decreased tissue viability. The combination of both supplements was not tolerated well as it lowered viability even further. On day 3, though, all supplements increased tissue viability, especially ascorbic acid. While doing so, BME with both supplements performed better than BME + insulin but worse than BME + vitamin C. Nevertheless, these differences were statistically not significant.

When serum-free NB-A groups were compared, the supplementation with insulin and/or ascorbic acid decreased viability. On day 1, especially the ascorbic acid supplement led to a drop in viability, while this was true for the insulin supplement on day 3. Vitamin C also lowered viability on day 5 in-vitro, but interestingly, insulin slightly increased it on this day. The combined supplementation with insulin and ascorbic acid decreased viability on all experimental days but its performance was in between insulin and vitamin C supplements except for day 7, which means

that it did not display an effect as negative as in BME-treated slices on days 1, 5 and 7.

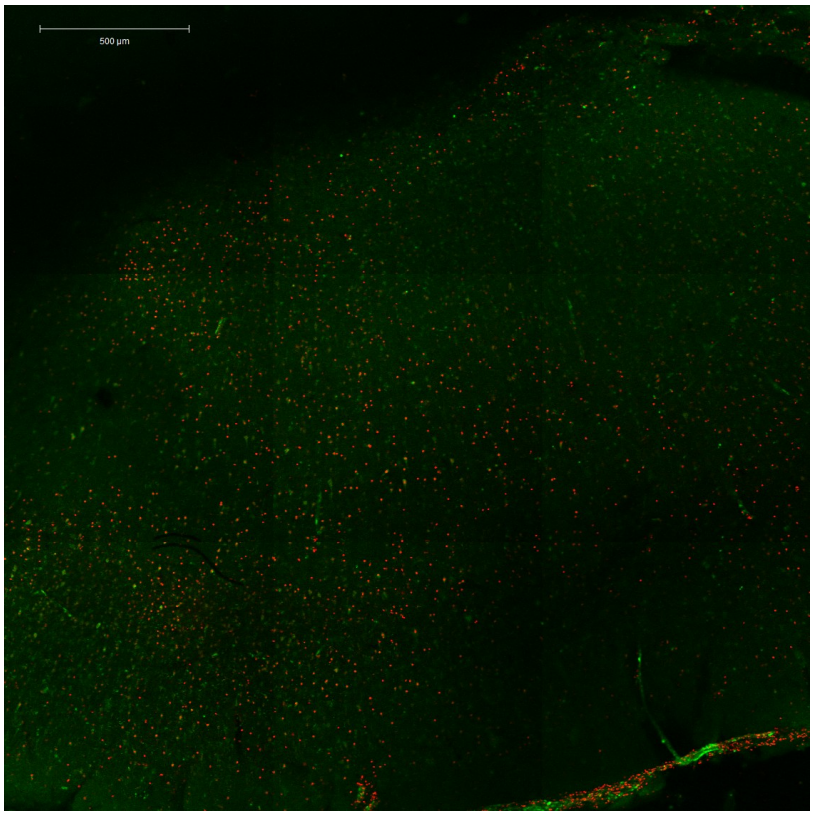

Figure 10: Cortical tissue on day 1 in-vitro.
Live cells are labelled green, dead cells are red.

3.3 Hippocampus

3.3.1 Day 1

On day 1 in-vitro, average tissue viability in the hippocampal region of the slices cultivated with serum-containing BME was 38.28% ± 11.56 (n=7) with a remarkably high variance, similar to that observed in in cortex (Figure 11). Slight and statistically not significant differences were observed in the average hippocampal viability in slices that were cultivated with BME medium supplemented with insulin and/or vitamin C (BME + insulin: 28.01% ± 7.37, n=4; BME + vitamin C: 32.86% ± 5.07, n=7; BME + vitamin C + insulin: 30.51% ± 3.23, n=7).

OTC cultured with serum-free NB-A showed higher levels of average viability (44.84% ± 5.85, n=10) than slices treated with serum-enriched BME groups, although the differences were not statistically significant. The difference was bigger in the slice cultures cultivated with insulin (49.06% ± 4.62, n=6) compared to those

with vitamin C supplementation (38.42% ± 3.91, n=7), where the average viability was lower than that of NB-A alone. Combined addition of insulin and vitamin C in NB-A, however, did not seem to have any detectable beneficial effect (34.01% ± 3.99, n=6). Notably, a statistically significant difference was also not observed in any group compared to the dead controls.

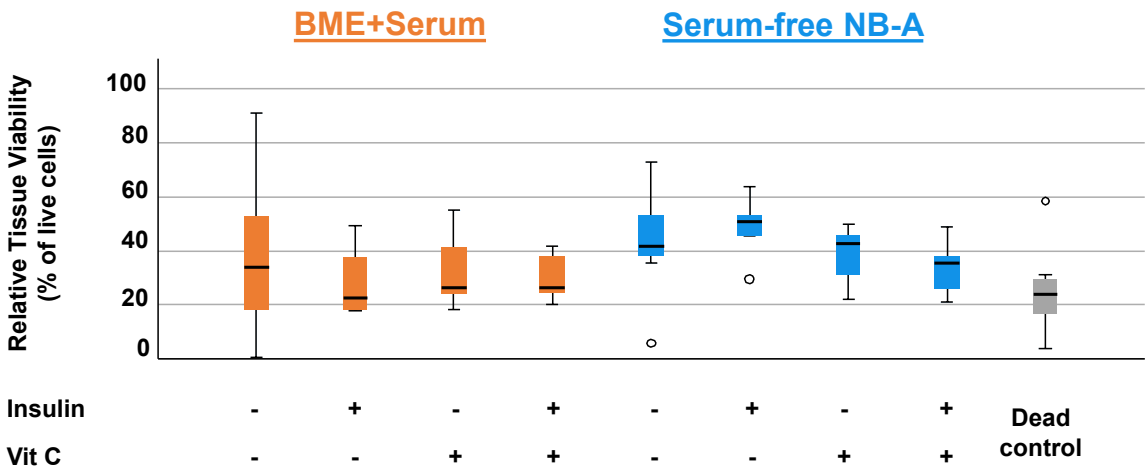

Figure 11: Hippocampus day 1, viability by medium.

3.3.2 Day 3

Hippocampal viability on day 3 in-vitro showed a profile similar to that of whole slices and cerebral cortex on day 3. The serum-containing BME group had an average viability of 17.26% ± 6.77 (n=8; Figure 12). The insulin-supplemented group did not show a notable difference (21.06% ± 7.37, n=7). Whilst viability in the vitamin C-supplemented BME group exhibited higher viability values (34.8% ± 4.33, n=6), although the differences were statistically not significant, combined supplementation with insulin and vitamin C seemed to be less beneficial than vitamin C alone (25.8% ± 6.41, n=5).

Slices cultivated with serum-free NB-A had generally higher viability levels compared to serum-containing BME, although the difference was statistically not significant (31.60% ± 4.99, n=9), while supplementation of NB-A with insulin and/or vitamin C did not seem to provide additional improvement to hippocampal viability on day 3 (NB-A + insulin: 26.23%, n=1; NB-A + vitamin C: 38.71% ± 14.79, n=3; NB-A + insulin + vitamin C: 30.38% ± 7.01, n=5). Statistically

significant differences were observed with NB-A (p=0.012) and BME + vit c (p=0.007) compared to dead control.

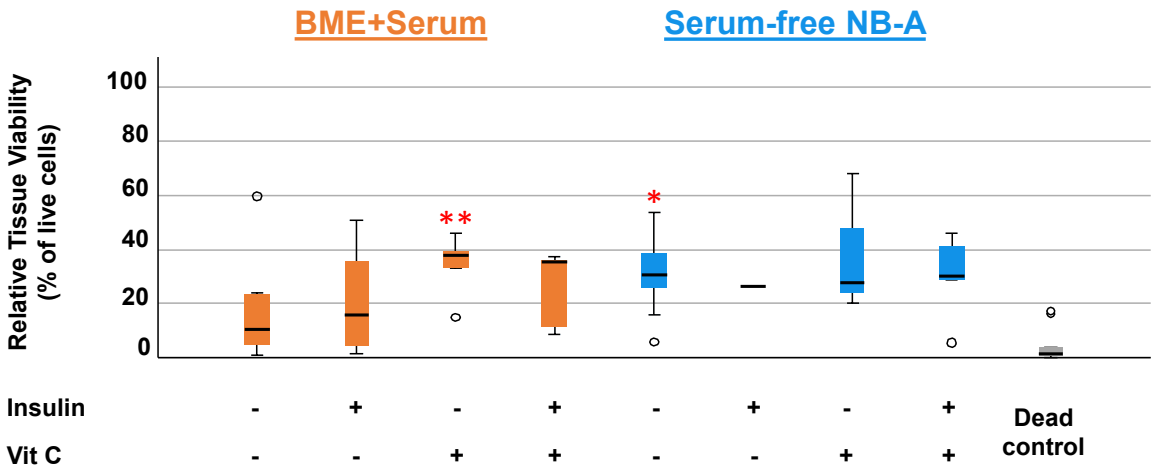

Figure 12: Hippocampus day 3, viability by medium.
Additionally, the differences between dead control and NB-A as well as between dead control and BME + vitamin C were statistically significant.

3.3.3 Day 5

On day 5, average viability in the slices cultured with serum-containing BME was 32.52% ± 22.22 (n=4), but the variance was extremely high, hindering a reliable estimation of the mean viability. The detected mean and median values in hippocampal viability in slices cultured with insulin (43.6% ± 29.67, n=2) or vitamin C supplement (51.46%, n=1) had extremely low sample sizes, and thus did not allow well-grounded interpretations.

Hippocampal viability in slices cultured with serum-free NB-A alone was lower than in the BME group (16.29% ± 12.12, n=3), which contrasts with the observations in cortical viability at the same timepoint. Consistent but limited improvement was observed in slices supplemented with vitamin C (24.56% ± 3.11, n=3). Pronounced variability and extremely low sample sizes in several groups did not allow drawing reliable inferences, as it hindered statistical assessment of the results.

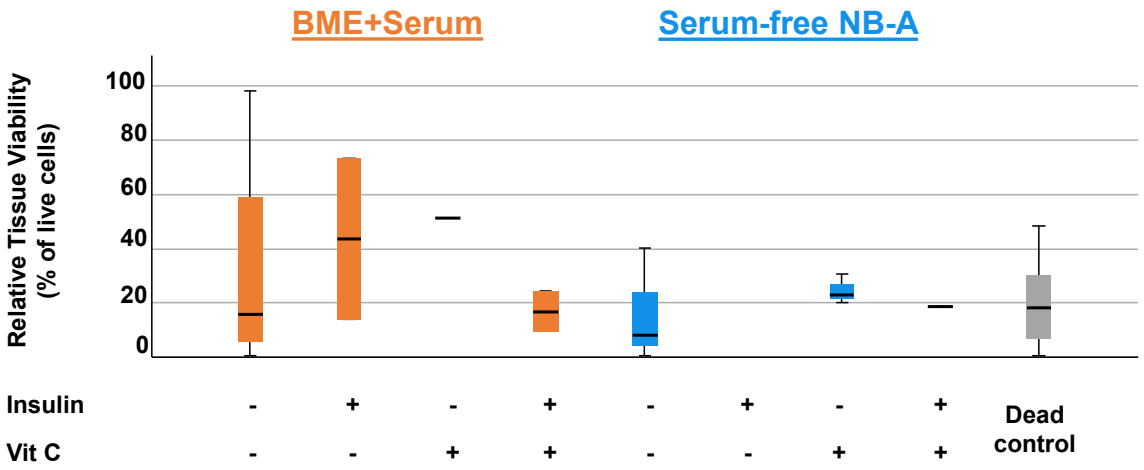

Figure 13: Hippocampus day 5, viability by medium.

3.3.4 Day 7

On day 7 in hippocampal tissue, average viability in OTC treated with serum-containing BME alone amounted to 42.33% ± 9.44 (n=4). Insulin alone (33.62% ± 3.74 SEM, n=5) or together with vitamin C (29.76% ± 9.44, n=4) did not yield a beneficial effect (Figure 14). Hardly any slices cultivated in a medium based on NB-A could be analysed because of slice exclusion.

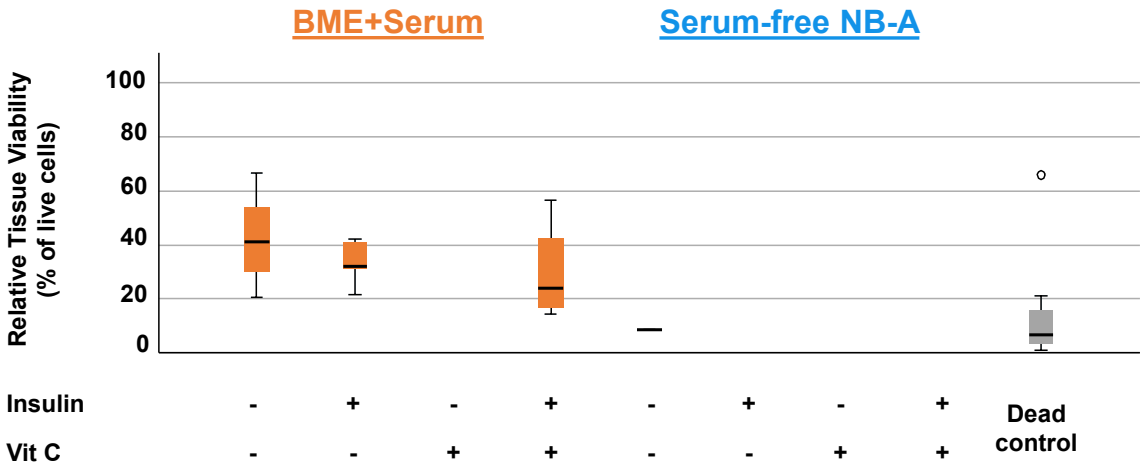

Figure 14: Hippocampus day 7, viability by medium.

3.3.5 Summary hippocampus

In hippocampal tissue (Figure 15), serum-free NB-A without supplements increased viability in comparison to serum-containing BME without supplements in the first

half of the experiment (days 1 and 3) but decreased it in the second half (not significant).

With regards to the used supplements in BME-based media, none increased tissue viability compared to BME alone on day 1. As for insulin, this supplement decreased viability also on day 7. In contrast, it increased cell survival on days 3 and 5 in-vitro. Ascorbic acid supplement, too, reduced viability in comparison to BME alone on day 1 and raised tissue viability on days 3 and 5. Nonetheless, ascorbic acid seemed to be better tolerated by hippocampal tissue as the observed reduction on day 1 was smaller and the increase in viability on days 3 and 5 was higher than when insulin was added. The combination of insulin and vitamin C supplements decreased viability to a similar extent as the other supplements on day 1, improved viability on day 3 and decreased it on days 5 and 7. Especially on day 5, the presence of both supplements in BME medium appeared to be detrimental as it performed markedly worse than insulin and vitamin C supplements. These differences were, however, not statistically significant.

Concerning supplements in slices that were being cultivated with NB-A, insulin improved viability in comparison to NB-A without supplements on day 1 and did not greatly change viability on day 3. AA reduced cell survival on day 1, slightly improved it on day 3 and increased it on the fifth day in-vitro, which is very similar to the influence of vitamin C on hippocampal regions in OTC cultivated in BME-based media. The observed effect of vitamin C + insulin supplement in NB-A on hippocampus was rather small: It slightly decreased cell retention on the first day in-vitro and showed cell survival close to the other NB-A-based media on the following days. These results were not statistically significant.

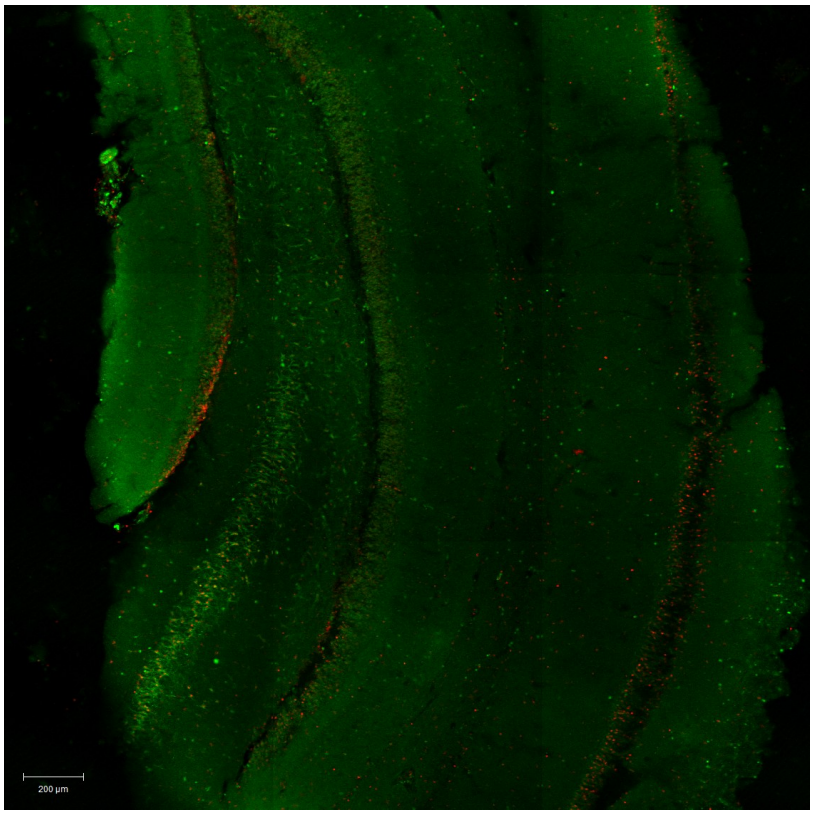

Figure 15: Hippocampal tissue on day 1 in-vitro.
Live cells are labelled green, dead cells are red.

3.4 Thalamus

3.4.1 Day 1

Average tissue viability in the thalamus region of the brain slices cultivated with serum-containing BME was 33.93% ± 7.88 (n=4; Figure 16). While cultures supplemented with insulin did not show any detectable difference, vitamin C-supplemented cultures had considerably higher viability (54.14% ± 6.78, n=5), although the difference was not statistically significant. Combined supplementation of vitamin C and insulin, however, did not have any beneficial effects (28.45% ± 7.94, n=6).

Average viability in the thalamus region of slices cultured in serum-free NB-A was comparable to the medium controls (35.61% ± 3.76, n=6). Insignificant slight improvements were detected with supplementation of insulin (49.45% ± 6.68, n=7) or vitamin C (41.85% ± 2.72, n=6). Combined supplementation of insulin and vitamin C seemed to be quite beneficial to the viability in thalamus on day 1 (55.19% ± 1.68, n=5), although the differences were statistically not significant. Significant differences compared with the dead control were observed in slices

cultured either with BME + vitamin C (p=0.028) or with NB-A + vitamin C + insulin (p=0.019).

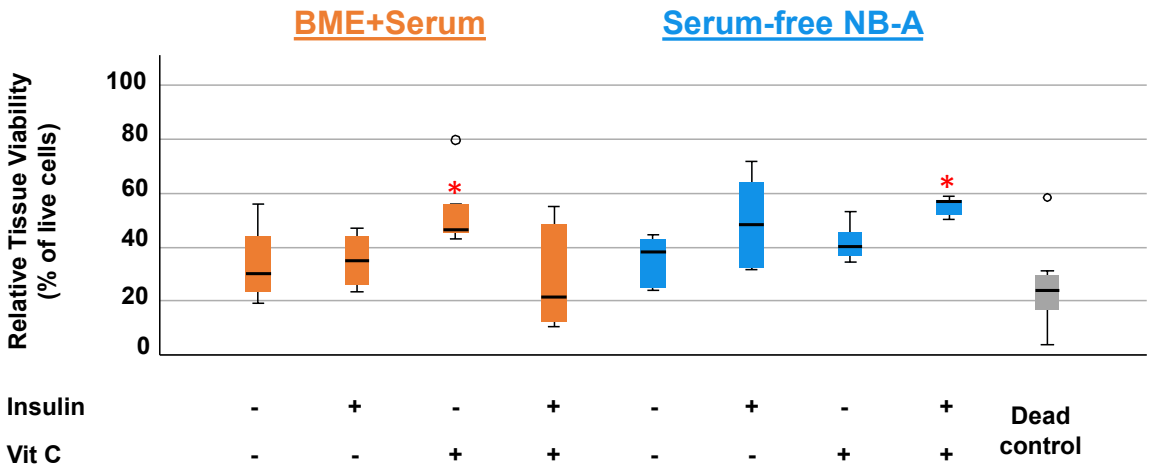

Figure 16: Thalamus day 1, viability by medium.
Additionally, dead control was significantly different from BME + vitamin C and NB-A + vitamin C + insulin groups.

3.4.2 Day 3

On day 3, slices cultivated with serum-containing BME showed an average viability of 28.18% ± 9.46 (n=8; Figure 17). Compared to this, lower viability levels were observed in the insulin-supplemented group (22.28% ± 11.34, n=7), whilst vitamin C alone or in combination with insulin resulted in improved levels of viability in the thalamic region on day 3 (BME + vitamin C: 40.12% ± 8.69, n=4; BME + vitamin C + insulin: 48.24% ± 7.45, n=7), although the differences were not statistically significant.

Slices cultured with serum-free NB-A medium had a consistently higher tissue viability in the thalamic region on day 3 than those with serum-containing BME did (49.06% ± 3.42, n=5). Insulin and/or vitamin C supplements, however, did not indicate any improvement, but instead resulted in tissue viability noticeably, but statistically not significantly, lower than which was observed without supplementation (NB-A + insulin: 46.44% ± 8.15, n=3; NB-A + vitamin C: 40.31% ± 7.63, n=4; NB-A + insulin + vitamin C: 36.82% ± 4.51, n=5). Statistically significant differences were detected in groups cultivated with BME + vitamin C + insulin (p=0.001), NB-A (p=0.003) and NB-A + insulin (p=0.045) compared with the dead controls.

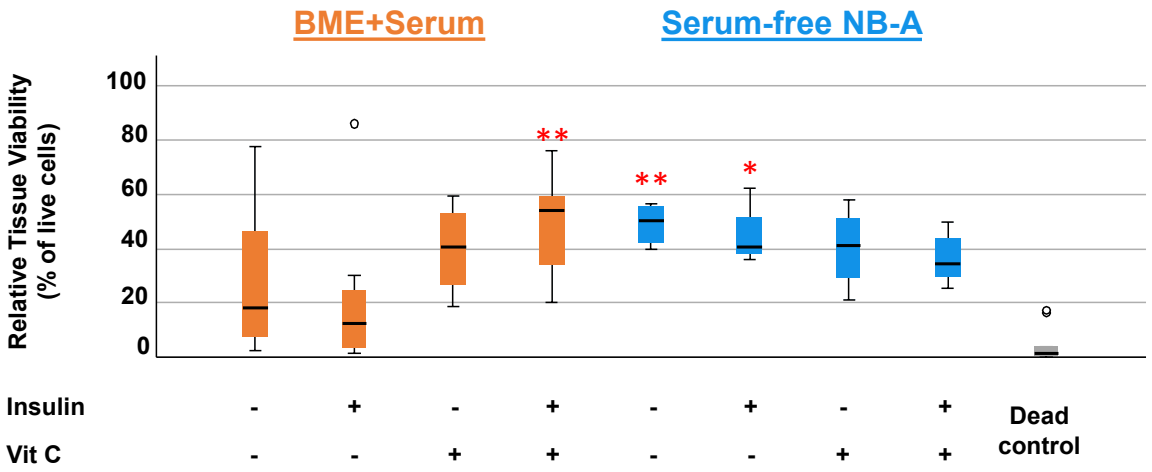

Figure 17: Thalamus day 3, viability by medium.
Additionally, dead control was significantly different from BME + vitamin C + insulin, NB-A + insulin and NB-A groups.

3.4.3 Day 5

At day 5 in-vitro, average tissue viability was measured above 40% in slices cultured with serum-containing BME alone or supplemented with insulin (Figure 18), yet both sample groups exhibited a high variability (BME: 45.73% ± 15.22, n=5; BME + insulin: 41.34% ± 7.77, n=8). The percentage of live cells in the vitamin C supplemented group, however, was substantially lower (11.50% ± 4.17, n=2), although statistical significance was not reached. Similar but more modest reductions were observed with combined supplementation of insulin and vitamin C (25.06% ± 6.86, n=5) suggesting that insulin and vitamin C exerted opposite effects.

Comparable to the observations in other brain regions, generally lower viability levels were observed at day 5 in-vitro also in the thalamic region with serum-free NB-A groups. Consistently lower percentages of live cells, in comparison to the BME group, was detected regardless of the supplemented medium additive (NB-A: 31.12% ± 4.57, n=7; NB-A + insulin: 25.79% ± 1.9, n=2; NB-A + vitamin C + insulin: 26.28% ± 2.13, n=2). Notably, the lowest viability levels were detected in the group cultured with vitamin C-supplemented NB-A, similar to what has been observed in the BME + vitamin C group (13.84% ± 4.13, n=3). Like in other brain regions, the percentage of live cells in all experimental groups was so low at this timepoint that they were statistically not significant from the dead controls.

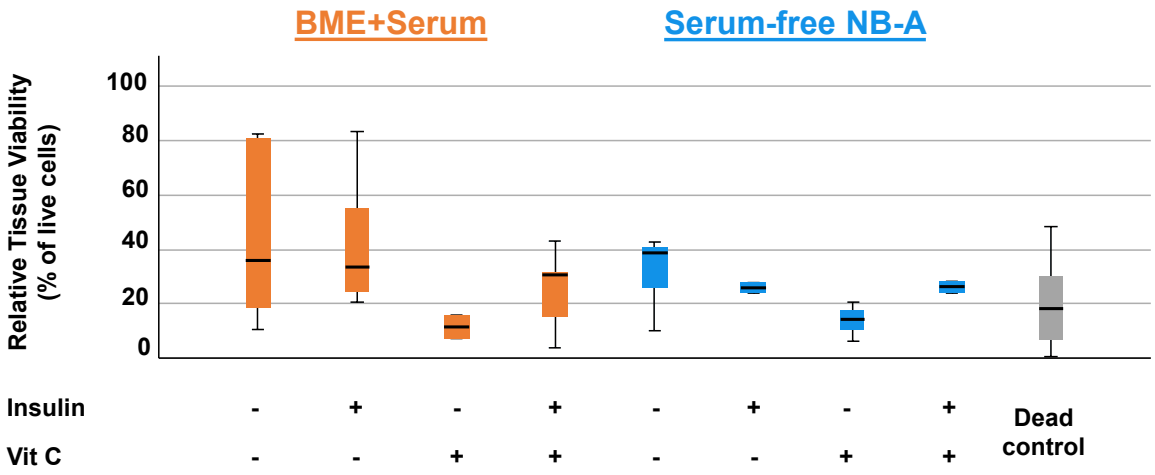

Figure 18: Thalamus day 5, viability by medium.

3.4.4 Day 7

As for thalamic tissue on day 7, the number of specimens was generally low. Average viability in the serum-containing BME group reached 46.48% ± 20.44 (n=2). Insulin supplementation in the BME medium did not have much of a beneficial effect (38.92% ± 6.09, n=3), but the lowest viability levels were detected in the vitamin C-supplemented slice with 9.08%, similar to day 5 but contrary to days 1 and 3, suggesting that the influence of vitamin C on culture viability changes time-dependently. Presence of insulin in the combined supplementation group seemed to balance vitamin C (23.28% ± 9.64, n=2) similar to the observations on day 5.

Serum-free NB-A groups exhibited rather lower levels of viability regardless of whether the medium was supplemented with insulin and/or vitamin C or not. Due to the low sample sizes in several groups, a statistical assessment of the differences does not have a high interpretational value.

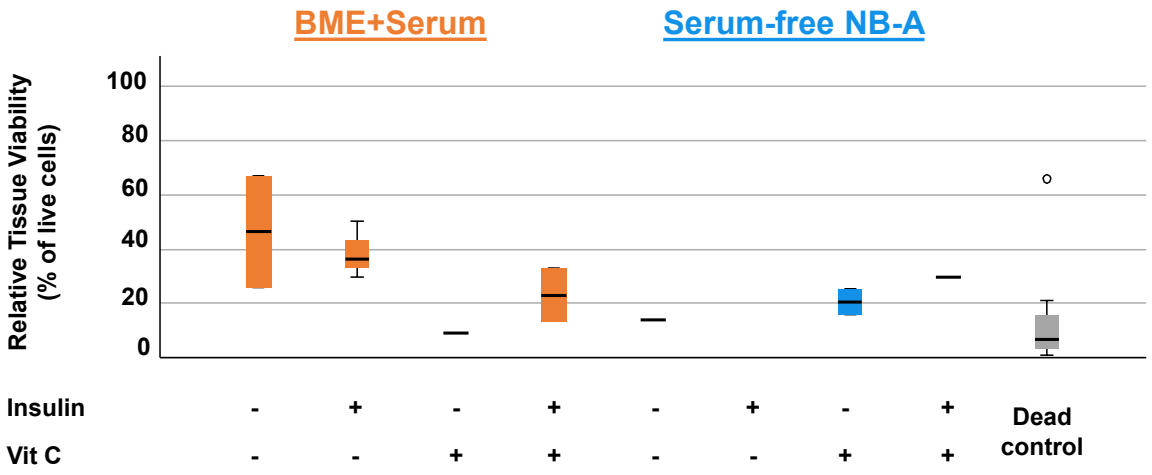

Figure 19: Thalamus day 7, viability by medium.

3.4.5 Summary thalamus

In thalamic tissue (Figure 20), NB-A medium slightly augmented cell survival in adult thalamus on day 1 and clearly on day 3 compared to BME. On days 5 and 7, however, NB-A decreased viability. No statistically significant difference was identified between these two groups.

In comparing BME without supplements to BME with insulin and/or AA, it is notable that insulin raised viability only after the first day in-vitro. At the other timepoints, insulin reduced the percentage of live cells. AA supplement greatly increased tissue survival during the first half of the experiment. On days 5 and 7 after transfer of tissue in-vitro, this changed and vitamin C considerably lowered cell viability. What was interesting was the influence of the combined insulin/AA supplement in BME media on adult thalamus. On the one hand, it markedly decreased viability on day 1, even though insulin and AA alone were able to increase it. On the other hand, it raised viability stronger than insulin and AA could on day 3. Both observations suggest a distinct effect of the combined supplement different from isolated insulin and AA on thalamus at the respective timepoints. Yet, they were in different directions. On days 5 and 7, however, this distinct effect was not observable as insulin + vitamin C performed in between insulin and AA alone. These reported differences were not statistically significant.

In NB-A medium, when the NB-A group was compared to the NB-A + insulin group, insulin supplementation was comparable to the addition of insulin to BME medium, since it increased viability after one day in-vitro and decreased it

afterwards. Vitamin C supplementation of NB-A, however, behaved different from AA supplementation in BME. Comparison of NB-A with NB-A + vitamin C revealed that AA enhanced cell survival on days 1 and 7, whereas it decreased viability on days 3 and 5 in-vitro. When the NB-A group was compared to the NB-A + vitamin C + insulin group, again, combination effects were observed. Both supplements together increased viability on days 1 and 7 (and performed better than their isolated counterparts insulin and AA) and decreased it on days 3 in-vitro (more than insulin and AA did). On day 5, too, it reduced viability but not as strongly as insulin or vitamin C. These reported differences were not statistically significant.

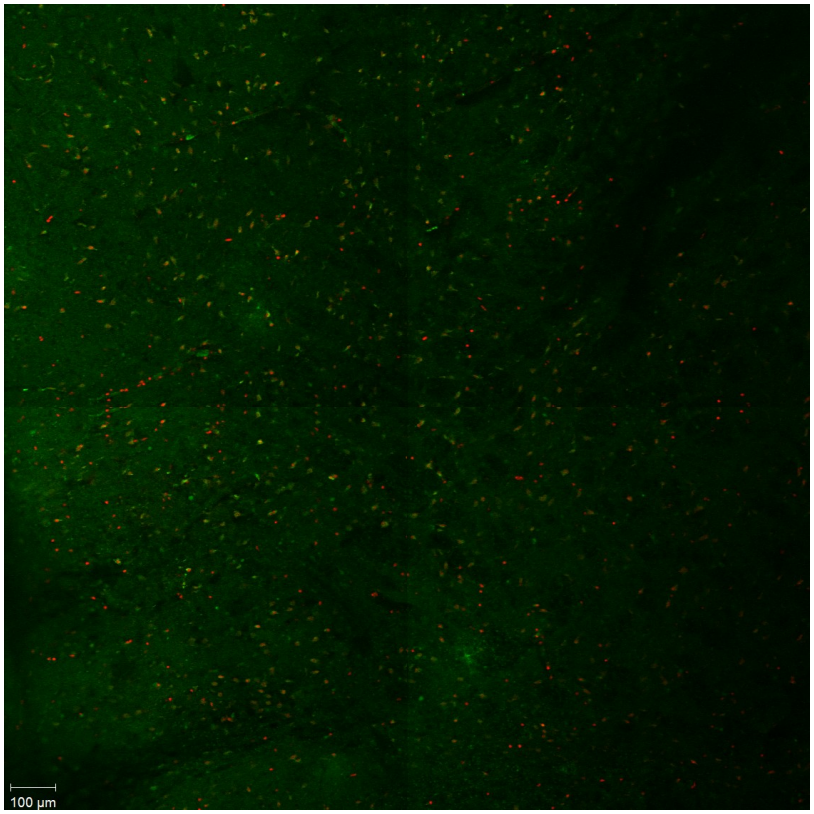

Figure 20: Thalamic tissue on day 1 in-vitro.
Live cells are labelled green, dead cells are red.

4 Discussion

Maintenance of adult rodent organotypic cultures is more challenging than culture of neonatal tissue, in particular due to the limited capacity of the adult tissue to stabilize cell counts. (26) This difference also raises the question whether results obtained from

neonatal brain slice cultures bear the validity for adult tissues. (58) Therefore, medium compositions that facilitate successful cultivation of adult brain slices would substantially contribute to the *Reduction* aim (3Rs) by reducing the number of required laboratory animals. (6,93) Adult brain slice cultures with improved viability could allow for a sufficient period for drug testing, ex-vivo investigation, the elucidation of molecular pathways, electrical stimulation, etc. and could therefore further research on adult brain pathologies like adult traumatic brain injury or neurodegenerative disorders.
In this study, the tissue viability of adult organotypic slice cultures in serum-free Neurobasal A medium was compared to a conventional BME-based medium with serum. Furthermore, the potential of single and combined supplementation of insulin and ascorbic acid to improve cell viability was assessed. Whether different brain subregions (cortex, hippocampus, thalamus) in a whole-brain slice could be supported by a common medium composition was also investigated.

4.1 Serum-free NB-A and serum-containing BME

The data gathered in this study did not indicate a difference in viability between serum-containing BME and serum-free NB-A media at day 1 in-vitro in any of the analysed brain regions. On day 3, however, big differences were observed particularly in cortical and whole-brain cell counts that were both statistically significant, but also in thalamic regions (not significant), where NB-A-containing groups had viability levels comparable to day 1, while BME groups showed reductions at day 3 compared to their day 1 counterparts. Similar results were observed in hippocampal regions (not significant), although to a much lesser extent. Improved viability - at least for a short time - is in line with the fact that the original formulation of NB-A for embryonic tissue was designed to optimize neuronal survival (64) and that it is suitable for the cultivation of several neuron populations. (94) Furthermore, NB-A is known to successfully support long-term survival of organotypic hippocampal slice cultures of adult mice over several weeks. (54) In addition, higher concentrations of energy sources in NB-A might explain higher viability (glucose: 25 mmol/L vs. approximately 17 mmol/L in BME; L-alanyl-L-glutamine 2 mmol/L vs. 1 mmol/L in BME). This beneficial influence of NB-A, however, was largely diminished at day 5 and 7 in viability of all three brain regions, probably because it does not support survival of glial cells, which presumably also

fulfil an important trophic support to the neurons in the cultivated slices like they do in-vivo. (95) Consistent with this presumption, increases in tissue viability seen exclusively in BME, but not NB-A groups at day 5 and 7 to the levels higher than that of day 3, could be explained by proliferation of glial cells. Whether this speculation holds true, requires further viability analysis of specific cell types at these timepoints.

4.2 Insulin

Insulin supplementation alone did not bring much of a benefit to the viability in neither of the three brain regions cultivated with serum-containing BME at any timepoint. On the contrary, it was associated with reductions in cortical viability at day 7. Similar results were obtained with NB-A groups, where insulin supplementation was not associated with any difference in either of the three regions at any timepoint, except lower viability levels in cortex at day 3. These differences were not statistically significant. In contrast to the data gathered here, other studies considered insulin to possess neuroprotective properties in organotypic brain culture of hippocampus (96,97), although not all subregions benefit from that. Tanaka and colleagues observed that the neuroprotective effect also depends on glucose concentration: Insulin was effective at low concentrations at 3 mmol/L, while in higher concentrations it was not. During glucose deprivation it was even detrimental. (96) The glucose levels in the here presented work were comparable to the higher levels in said study. This would explain the lack of effectiveness provided by insulin in hippocampus and possibly in other subregions, too. Another reason could be the older age of the tissue used here, since it could react differently to an insulin stimulus than younger tissue. An additional cause for the observed ineffectiveness of insulin could be the high variability between slices. Furthermore, it must be mentioned that baseline levels of insulin (before addition of the supplement) could have been different in serum-free NB-A- and serum-containing BME-based media, since both contained insulin, but its concentration in serum and NB-A was neither measured nor disclosed by the manufacturers. Given the lack of data of insulin on adult OTC viability, more studies are needed to better understand its effects.

4.3 Ascorbic acid

Vitamin C, unlike insulin, supplementation resulted in differential influence among the regions at different timepoints. Improved viability was observed with vitamin C-supplemented BME in cortex and hippocampus exclusively at day 3 – a timepoint characterized with strong reductions compared to day 1 in BME without supplements. Notably, this beneficial influence of vitamin C was detectable in thalamic regions both at day 1 and 3. However, such beneficial effects of vitamin C were not detectable with the serum-free NB-A media in either of the three regions analysed at any timepoint. Notably, all these differences were not statistically significant. In OTC obtained from younger animals, AA seems to support tissue survival through the reduction of oxidative stress. (98) However, another study, which subjected adult brain slices to H2O2-induced injury, failed to reduce cell death by AA supplementation, which is rather in agreement with the data presented here in this work. This observation was explained by reduced AA incorporation in mature brain tissue and dose-dependent saturation of a sodium-ascorbate co-transporter failing to increase vitamin C content of the tissue. (99) All these findings imply that tissue age might be a critical factor for AA supplementation in OTC. In contrast, a study aiming to increase culture time of adult OTC succeeded in prolonging lifespan by a day at a low viability level. (51) This suggests that AA has at least weak survival-promoting effects in adult OTC, which was implicitly recognisable in the here presented data. Another survey found that merely weeklong vitamin C supplementation exerted significant effects on adult OTC viability by increasing gene expression of antioxidant factors. (79) Therefore, the observation time in this study might have been too short to identify a significant effect. In addition, another explanation for the insufficient impact of AA in this study might be the high variability between slices that could have masked or attenuated minor effects. Nonetheless, more research is needed to further clarify the effect of AA on adult OTC.

4.4 Ascorbic acid in combination with insulin

Beneficial influence of the combined supplementation of insulin and vitamin C in BME was observed rather at day 3 in-vitro, a timepoint when BME without supplements exhibited lower viability levels compared to day 1. These results were

similar to those of vitamin C alone, suggesting that the outcome of the combined supplementation is rather determined by vitamin C. This presumption, however, does not hold true at day 1, when three different influences could be observed in three different regions: (i) detrimental in cortex, contrasting with the ineffectiveness of each additive alone, (ii) ineffective in hippocampus, similar to each additive alone or (iii) ineffective in thalamus, approximating to insulin alone and in contrast to the beneficial effect of vitamin C alone. Such discrepancies were also observed with NB-A groups, but in different ways: (i) cortex: ineffective at day 1, similar to each additive alone, while detrimental at day 3, approximating to insulin alone, (ii) hippocampus: less desirable than each additive alone at day 1, while ineffective at day 3, similar to each additive alone; (iii) thalamus: rather beneficial at day 1, contrasting with the ineffectiveness of each additive alone, while less desirable at day 3 than each additive alone. Of note, all these differences were not statistically significant. The only other study investigating this combination found that the lifespan of adult hippocampal OTC could be extended by a day showing a viability level of 50% on day 4 in-vitro, which was considerably higher than with supplementation of AA alone. (51) This finding is not in agreement with the findings presented in this study. This might be explained by methodological differences, since this group, i.e., measured functional neuronal viability but not overall cell viability. It is possible (yet speculative) to assume, that neurons might benefit from the combined supplement, whereas other cell types like astrocytes might not. Adding to that, variability was very high in the here presented work, and thus might have impaired statistical validity.

Taken together, the here presented data suggests that the influence of insulin-vitamin C co-supplementation could not be straightforwardly inferred from the separate effects of each additive, but it is rather diversified with respect to the timepoint, analysed brain region as well as the selected base medium. More research is needed to clarify the effect of the combined supplement on adult OTC.

4.5 Limitations

Several important limitations apply in this study. First, this study was designed to investigate cell survival irrespective of cell type. Thus, no inferences can be drawn from the data about survival of cell types such as neurons, astrocytes, microglia etc.

Second, low sample sizes and severe group size differences among the experimental groups due to slice exclusion reduced the statistical power. Third, variability between slices was very high. This was partly caused by the utilization of new slices at each timepoint instead of repeated measurements. Another portion could be related to issues such as 1) uneven lighting of the images, 2) the occurrence of cells stained by both calcein and EthD-1 (probably due to cell stress during the incubation time outside the incubator leading to a drop in temperature, while medium was also removed), 3) high background staining and low image resolution in calcein-stained images and 4) partly inaccurate object identification in EthD-1-stained slices, since the used algorithm sometimes identified a large confluent cell across an area that was actually consisting of multiple cells. This problem was mitigated by "Watershed" and "Fill holes" commands but could not be eliminated.

4.6 Outlook, recommendations, and conclusion

This work has revealed that single medium supplements can influence viability in adult OTC. However, according to the data, the chosen additives did not substantially increase viability to reliably ensure longer survival and further experimentation. Other factors like brain region, timepoint and the used base medium heavily impacted tissue survival. Based on the gathered data, what could be improved in future studies? First, further research is needed to fully elucidate the effects of serum-free NB-A, insulin, AA or both on adult OTC. Second, if any medium supplement aiming to improve viability is investigated, sample size should be increased and variability between slices reduced (i.e., by repeated measurements of the same slices). Third, when cortex or a whole brain is investigated on day 3, NB-A is recommended when optimization of viability is desired. Fourth, frequent medium adjustments during cultivation could be justified. For example, in adult cortical OTC, serum-free NB-A medium could be employed until day 3, and then switched to serum-containing BME. Alternatively, it might be advisable to 1) test if increased concentrations of glucose or glutamine are beneficial for adult OTC, 2) to concentrate on tailoring a highly specific medium optimized to the brain region and time point of interest as well as to 3) focus on other environmental factors such as temperature, nutrient flow, or growth factors.

In conclusion, the data gathered in this study suggests an advantage of serum-free Neurobasal A medium over formulations containing serum on day 3 in-vitro in whole-brain and cortical tissue. Furthermore, it provides clues, which suggest that medium supplements, for instance ascorbic acid, are worth further investigation for certain subregions on certain days but probably not as a generic tool to improve cell survival without other contributing factors.

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Appendix

	A	B	C	D	E	F	G	H	I	J
1	Brain region	day	Levene's test (based on mean)	ANOVA	Welch ANOVA	(Welch) ANOVA p-value	Post-hoc test for (Welch) ANOVA	Kruskal-Wallis	Kruskal-Wallis p-value	number of comparisons
2	Total	1	p=0.014		all other groups	p=0.000	Games-Howell	BME + vit c, neurobasal a + insulin	p=0.011	15
3	Total	3	p=0.068	all other groups	-	0.026	Gabriel	dead control, BME + vit c + insulin	0.000	15
4	Total	5	p=0.207	all other groups	-	p=0.663	-	BME + vit c	0.494	-
5	Total	7	p=0.540	medium ctrl, BME + insulin, BME + vit c + insulin	-	p=0.239	-	all other groups	p=0.062	-
6	Cortex	1	p=0.095	all groups	-	p=0.044	Gabriel	-	-	-
7	Cortex	3	p=0.861	all other groups	-	p=0.022	Gabriel	medium control, dead control	p=0.000	15
8	Cortex	5	p=0.006	-	all other groups	p=0.112	-	BME + vit c, BME + vit c + insulin	p=0.615	-
9	Cortex	7	p=985	medium control, BME + insulin, BME + vit C + insulin	-	p=0.090	-	dead control, Neurobasal A, BME + vit c, Neurobasal A + vit C, Neurobasal A + vit C + insulin	p=0.141	-
10	Hippocampus	1	p=0.097	all groups	-	p=0.171	-	-	-	-
11	Hippocampus	3	p=0.203	all other groups	-	p=0.505	-	dead control medium ctrl BME + vit c +insulin neurobasal a + insulin	p=0.006	26
12	Hippocampus	5	p=0.046	-	all other groups	p=0.843	-	BME + insulin, BME + vit c, BME + vit c + insulin, neurobasal a + vit c + insulin	p=0.812	-
13	Hippocampus	7	p=0.523	all other groups	-	p=0.523	-	dead control, neurobasal a	p=0.055	-
14	Thalamus	1	p=0.093	all groups	-	p=0.003	Gabriel	-	-	-
15	Thalamus	3	p=0.080	all other groups	-	p=0.413	-	dead control, BME + insulin	p=0.001	15
16	Thalamus	5	p=0.007	-	all other groups	p=0.070	-	BME + vit c neurobasal a + insulin neurobasal a + vit c + insulin	p=0.168	-
17	Thalamus	7	-	-	-	-	-	all groups	p=0.182	-

Table 1: Utilized statistical tests by group and their results.
The number of comparisons was relevant for Bonferroni correction of pairwise analysis after a significant Kruskal-Wallis test.

Post-hoc test

Abhängige Variable:

group			Mittelwertdifferenz (I-J)	Std.-Fehler	Sig.	95% Konfidenzintervall Untergrenze	95% Konfidenzintervall Obergrenze
Games-Howell	dead ctrl	medium ctrl	-19,90031%*	5,26999%	0,009	-36,3396%	-3,4610%
		BME + insulin	-16,84265%*	4,07674%	0,003	-29,4626%	-4,2226%
		neurobasal a	-26,94171%*	4,31157%	0,000	-40,4110%	-13,4724%
		BME + vit c + insulin	-16,59994%*	3,75567%	0,001	-28,2122%	-4,9877%
		neurobasal a + vit c	-17,46326%*	4,51209%	0,009	-31,6910%	-3,2355%
		neurobasal a + vit c + insulin	-20,15606%*	3,50667%	0,000	-31,0657%	-9,2464%
	medium ctrl	dead ctrl	19,90031%*	5,26999%	0,009	3,4610%	36,3396%
		BME + insulin	3,05766%	5,57766%	0,998	-14,2490%	20,3643%
		neurobasal a	-7,04140%	5,75153%	0,881	-24,8887%	10,8059%
		BME + vit c + insulin	3,30036%	5,34747%	0,996	-13,3668%	19,9675%
		neurobasal a + vit c	2,43704%	5,90334%	1,000	-15,9069%	20,7810%
		neurobasal a + vit c + insulin	-0,25576%	5,17563%	1,000	-16,4829%	15,9714%
	BME + insulin	dead ctrl	16,84265%*	4,07674%	0,003	4,2226%	29,4626%
		medium ctrl	-3,05766%	5,57766%	0,998	-20,3643%	14,2490%
		neurobasal a	-10,09906%	4,68264%	0,342	-24,6704%	4,4723%
		BME + vit c + insulin	0,24270%	4,17642%	1,000	-12,7103%	13,1957%
		neurobasal a + vit c	-0,62062%	4,86790%	1,000	-15,8629%	14,6217%
		neurobasal a + vit c + insulin	-3,31342%	3,95401%	0,979	-15,6620%	9,0352%
	neurobasal a	dead ctrl	26,94171%*	4,31157%	0,000	13,4724%	40,4110%
		medium ctrl	7,04140%	5,75153%	0,881	-10,8059%	24,8887%
		BME + insulin	10,09906%	4,68264%	0,342	-4,4723%	24,6704%
		BME + vit c + insulin	10,34176%	4,40594%	0,251	-3,4272%	24,1107%
		neurobasal a + vit c	9,47844%	5,06619%	0,512	-6,4012%	25,3581%
		neurobasal a + vit c + insulin	6,78564%	4,19572%	0,673	-6,4354%	20,0067%
	BME + vit c + insulin	dead ctrl	16,59994%*	3,75567%	0,001	4,9877%	28,2122%
		medium ctrl	-3,30036%	5,34747%	0,996	-19,9675%	13,3668%
		BME + insulin	-0,24270%	4,17642%	1,000	-13,1957%	12,7103%
		neurobasal a	-10,34176%	4,40594%	0,251	-24,1107%	3,4272%
		neurobasal a + vit c	-0,86332%	4,60235%	1,000	-15,3642%	13,6375%
		neurobasal a + vit c + insulin	-3,55612%	3,62207%	0,955	-14,8785%	7,7663%
	neurobasal a + vit c	dead ctrl	17,46326%*	4,51209%	0,009	3,2355%	31,6910%
		medium ctrl	-2,43704%	5,90334%	1,000	-20,7810%	15,9069%
		BME + insulin	0,62062%	4,86790%	1,000	-14,6217%	15,8629%
		neurobasal a	-9,47844%	5,06619%	0,512	-25,3581%	6,4012%
		BME + vit c + insulin	0,86332%	4,60235%	1,000	-13,6375%	15,3642%
		neurobasal a + vit c + insulin	-2,69280%	4,40152%	0,996	-16,6924%	11,3068%
	neurobasal a + vit c + insulin	dead ctrl	20,15606%*	3,50667%	0,000	9,2464%	31,0657%
		medium ctrl	0,25576%	5,17563%	1,000	-15,9714%	16,4829%
		BME + insulin	3,31342%	3,95401%	0,979	-9,0352%	15,6620%
		neurobasal a	-6,78564%	4,19572%	0,673	-20,0067%	6,4354%
		BME + vit c + insulin	3,55612%	3,62207%	0,955	-7,7663%	14,8785%
		neurobasal a + vit c	2,69280%	4,40152%	0,996	-11,3068%	16,6924%

*. Die Mittelwertdifferenz ist in Stufe 0.05 signifikant.

Table 2: Total day 1 post-hoc test following significant Welch's ANOVA. Significant p-values are highlighted in green.

Paarweise Vergleiche von group

Sample 1-Sample 2	Teststatistik	Standardfehler	Standardteststatistik	Sig.	Anp. Sig. Neu[a]
BME + vit c + insulin-BME + vit c	23,333	9,416	2,478	0,013	0,198196638
BME + vit c + insulin-neurobasal a + insulin	-29,667	9,416	-3,151	0,002	0,024443178
dead ctrl-BME + vit c	-21,292	8,808	-2,417	0,016	0,234560195
dead ctrl-neurobasal a + insulin	-27,625	8,808	-3,136	0,002	0,025663816
BME + insulin-BME + vit c	-9,067	9,876	-0,918	0,359	5,378810986
BME + insulin-neurobasal a + insulin	-15,400	9,876	-1,559	0,119	1,78369603
neurobasal a + vit c-BME + vit c	9,000	9,416	0,956	0,339	5,087677857
neurobasal a + vit c-neurobasal a + insulin	15,333	9,416	1,628	0,103	1,551657571
neurobasal a + vit c + insulin-BME + vit c	4,000	9,416	0,425	0,671	10,06478752
neurobasal a + vit c + insulin-neurobasal a + insulin	10,333	9,416	1,097	0,272	4,087076569
medium ctrl-BME + vit c	-1,810	9,074	-0,199	0,842	12,62897155
medium ctrl-neurobasal a + insulin	-8,143	9,074	-0,897	0,370	5,542534722
BME + vit c-neurobasal a	1,167	9,416	0,124	0,901	13,52093275
BME + vit c-neurobasal a + insulin	-6,333	9,416	-0,673	0,501	7,518096621
neurobasal a-neurobasal a + insulin	-5,167	9,416	-0,549	0,583	8,74823143

Jede Zeile prüft die Nullhypothese, dass die Verteilungen in Stichprobe 1 und Stichprobe 2 gleich sind.
Asymptotische Signifikanzen (zweiseitige Tests) werden angezeigt. Das Signifikanzniveau ist ,050.

a. Signifikanzwerte wurden durch Bonferroni-Korrektur für mehrere Tests angepasst.

Table 3: Kruskal-Wallis' test for total day 1 with Bonferroni correction (15 comparisons). Significant, corrected p-values are highlighted in green.

Post-hoc test

Abhängige Variable:

group			Mittelwertdifferenz (I-J)	Std.-Fehler	Sig.	95% Untergrenze	95% Obergrenze
Gabriel	medium ctrl	BME + insulin	-2,45302%	8,13088%	1,000	-29,0305%	24,1245%
		neurobasal a	-28,14026%*	8,46289%	0,043	-55,7825%	-0,4980%
		BME + vit c	-14,91904%	9,53431%	0,901	-45,7872%	15,9491%
		neurobasal a + insulin	-20,72872%	9,53431%	0,481	-51,5968%	10,1394%
		neurobasal a + vit c	-20,85674%	8,90694%	0,373	-49,8690%	8,1555%
		neurobasal a + vit c + insulin	-13,98047%	8,46289%	0,865	-41,6227%	13,6618%
	BME + insulin	medium ctrl	2,45302%	8,13088%	1,000	-24,1245%	29,0305%
		neurobasal a	-25,68725%	8,46289%	0,088	-53,3295%	1,9550%
		BME + vit c	-12,46602%	9,53431%	0,978	-43,3341%	18,4021%
		neurobasal a + insulin	-18,27570%	9,53431%	0,678	-49,1438%	12,5924%
		neurobasal a + vit c	-18,40372%	8,90694%	0,573	-47,4159%	10,6085%
		neurobasal a + vit c + insulin	-11,52746%	8,46289%	0,970	-39,1697%	16,1148%
	neurobasal a	medium ctrl	28,14026%*	8,46289%	0,043	0,4980%	55,7825%
		BME + insulin	25,68725%	8,46289%	0,088	-1,9550%	53,3295%
		BME + vit c	13,22123%	9,81898%	0,972	-18,7116%	45,1541%
		neurobasal a + insulin	7,41154%	9,81898%	1,000	-24,5213%	39,3444%
		neurobasal a + vit c	7,28352%	9,21102%	1,000	-22,7934%	37,3605%
		neurobasal a + vit c + insulin	14,15979%	8,78236%	0,885	-14,5472%	42,8668%
	BME + vit c	medium ctrl	14,91904%	9,53431%	0,901	-15,9491%	45,7872%
		BME + insulin	12,46602%	9,53431%	0,978	-18,4021%	43,3341%
		neurobasal a	-13,22123%	9,81898%	0,972	-45,1541%	18,7116%
		neurobasal a + insulin	-5,80969%	10,75615%	1,000	-40,9684%	29,3491%
		neurobasal a + vit c	-5,93770%	10,20418%	1,000	-39,2405%	27,3651%
		neurobasal a + vit c + insulin	0,93856%	9,81898%	1,000	-30,9943%	32,8714%
	neurobasal a + insulin	medium ctrl	20,72872%	9,53431%	0,481	-10,1394%	51,5968%
		BME + insulin	18,27570%	9,53431%	0,678	-12,5924%	49,1438%
		neurobasal a	-7,41154%	9,81898%	1,000	-39,3444%	24,5213%
		BME + vit c	5,80969%	10,75615%	1,000	-29,3491%	40,9684%
		neurobasal a + vit c	-0,12802%	10,20418%	1,000	-33,4309%	33,1748%
		neurobasal a + vit c + insulin	6,74825%	9,81898%	1,000	-25,1846%	38,6811%
	neurobasal a + vit c	medium ctrl	20,85674%	8,90694%	0,373	-8,1555%	49,8690%
		BME + insulin	18,40372%	8,90694%	0,573	-10,6085%	47,4159%
		neurobasal a	-7,28352%	9,21102%	1,000	-37,3605%	22,7934%
		BME + vit c	5,93770%	10,20418%	1,000	-27,3651%	39,2405%
		neurobasal a + insulin	0,12802%	10,20418%	1,000	-33,1748%	33,4309%
		neurobasal a + vit c + insulin	6,87627%	9,21102%	1,000	-23,2007%	36,9532%
	neurobasal a + vit c + insulin	medium ctrl	13,98047%	8,46289%	0,865	-13,6618%	41,6227%
		BME + insulin	11,52746%	8,46289%	0,970	-16,1148%	39,1697%
		neurobasal a	-14,15979%	8,78236%	0,885	-42,8668%	14,5472%
		BME + vit c	-0,93856%	9,81898%	1,000	-32,8714%	30,9943%
		neurobasal a + insulin	-6,74825%	9,81898%	1,000	-38,6811%	25,1846%
		neurobasal a + vit c	-6,87627%	9,21102%	1,000	-36,9532%	23,2007%

*. Die Mittelwertdifferenz ist in Stufe 0.05 signifikant.

Table 4: Total day 3 post-hoc test following significant ANOVA. Significant p-values are highlighted in green.

Paarweise Vergleiche von group

Sample 1-Sample 2	Teststatistik	Standardfehler	Standardteststatistik	Sig.	Anp. Sig. Neu[a]
dead ctrl-medium ctrl	-14,444	7,928	-1,822	0,068	1,027062062
dead ctrl-BME + insulin	-16,302	7,928	-2,056	0,040	0,59653608
dead ctrl-neurobasal a + vit c + ins	-25,111	8,292	-3,029	0,002	0,03686396
dead ctrl-BME + vit c	-26,194	9,454	-2,771	0,006	0,08388474
dead ctrl-BME + vit c + insulin	-28,611	8,292	-3,451	0,001	0,008389201
dead ctrl-neurobasal a + insulin	-29,694	9,454	-3,141	0,002	0,025256278
dead ctrl-neurobasal a + vit c	-30,444	8,775	-3,469	0,001	0,007822398
dead ctrl-neurobasal a	-36,278	8,292	-4,375	0,000	0,000181928
medium ctrl-BME + vit c + insulin	-14,167	8,753	-1,619	0,106	1,583078483
BME + insulin-BME + vit c + insulin	-12,310	8,753	-1,406	0,160	2,394112962
neurobasal a + vit c + ins-BME + vit c + insulin	3,500	9,083	0,385	0,700	10,49981275
BME + vit c-BME + vit c + insulin	-2,417	10,155	-0,238	0,812	12,17848536
BME + vit c + insulin-neurobasal a + insulin	-1,083	10,155	-0,107	0,915	13,72565117
BME + vit c + insulin-neurobasal a + vit c	-1,833	9,526	-0,192	0,847	12,7108449
BME + vit c + insulin-neurobasal a	7,667	9,083	0,844	0,399	5,979435752

Jede Zeile prüft die Nullhypothese, dass die Verteilungen in Stichprobe 1 und Stichprobe 2 gleich sind.
Asymptotische Signifikanzen (zweiseitige Tests) werden angezeigt. Das Signifikanzniveau ist ,050.

a. Signifikanzwerte werden von der Bonferroni-Korrektur für mehrere Tests angepasst.

Table 5: Kruskal-Wallis' test for total day 3 with Bonferroni correction (15 comparisons). Significant, corrected p-values are highlighted in green.

Post-hoc test

Abhängige Variable:

group			Mittelwertdifferenz (I-J)	Std.-Fehler	Sig.	95% Untergrenze	95% Obergrenze
Gabriel	dead ctrl	medium ctrl	-24,45504%	8,24656%	0,143	-52,2088%	3,2987%
		BME + insulin	-17,72834%	8,70505%	0,757	-46,9017%	11,4450%
		neurobasal a	-19,35633%	7,90280%	0,436	-46,0068%	7,2941%
		BME + vit c	-15,49017%	8,24656%	0,867	-43,2439%	12,2636%
		BME + vit c + insulin	1,12382%	8,24656%	1,000	-26,6299%	28,8776%
		neurobasal a + insulin	-16,81184%	8,24656%	0,762	-44,5656%	10,9419%
		neurobasal a + vit c	-5,57370%	8,24656%	1,000	-33,3275%	22,1801%
		neurobasal a + vit c + insulin	-12,36385%	8,24656%	0,987	-40,1176%	15,3899%
	medium ctrl	dead ctrl	24,45504%	8,24656%	0,143	-3,2987%	52,2088%
		BME + insulin	6,72670%	9,24624%	1,000	-24,4393%	37,8927%
		neurobasal a	5,09871%	8,49526%	1,000	-23,5444%	33,7418%
		BME + vit c	8,96486%	8,81594%	1,000	-20,7815%	38,7113%
		BME + vit c + insulin	25,57886%	8,81594%	0,170	-4,1675%	55,3252%
		neurobasal a + insulin	7,64320%	8,81594%	1,000	-22,1032%	37,3896%
		neurobasal a + vit c	18,88134%	8,81594%	0,686	-10,8650%	48,6277%
		neurobasal a + vit c + insulin	12,09119%	8,81594%	0,996	-17,6552%	41,8376%
	BME + insulin	dead ctrl	17,72834%	8,70505%	0,757	-11,4450%	46,9017%
		medium ctrl	-6,72670%	9,24624%	1,000	-37,8927%	24,4393%
		neurobasal a	-1,62799%	8,94100%	1,000	-31,6907%	28,4347%
		BME + vit c	2,23816%	9,24624%	1,000	-28,9278%	33,4041%
		BME + vit c + insulin	18,85216%	9,24624%	0,764	-12,3138%	50,0181%
		neurobasal a + insulin	0,91650%	9,24624%	1,000	-30,2495%	32,0825%
		neurobasal a + vit c	12,15464%	9,24624%	0,998	-19,0113%	43,3206%
		neurobasal a + vit c + insulin	5,36449%	9,24624%	1,000	-25,8015%	36,5304%
	neurobasal a	dead ctrl	19,35633%	7,90280%	0,436	-7,2941%	46,0068%
		medium ctrl	-5,09871%	8,49526%	1,000	-33,7418%	23,5444%
		BME + insulin	1,62799%	8,94100%	1,000	-28,4347%	31,6907%
		BME + vit c	3,86615%	8,49526%	1,000	-24,7769%	32,5092%
		BME + vit c + insulin	20,48015%	8,49526%	0,466	-8,1629%	49,1232%
		neurobasal a + insulin	2,54449%	8,49526%	1,000	-26,0986%	31,1876%
		neurobasal a + vit c	13,78263%	8,49526%	0,968	-14,8605%	42,4257%
		neurobasal a + vit c + insulin	6,99248%	8,49526%	1,000	-21,6506%	35,6356%
	BME + vit c	dead ctrl	15,49017%	8,24656%	0,867	-12,2636%	43,2439%
		medium ctrl	-8,96486%	8,81594%	1,000	-38,7113%	20,7815%
		BME + insulin	-2,23816%	9,24624%	1,000	-33,4041%	28,9278%
		neurobasal a	-3,86615%	8,49526%	1,000	-32,5092%	24,7769%
		BME + vit c + insulin	16,61400%	8,81594%	0,867	-13,1324%	46,3604%
		neurobasal a + insulin	-1,32166%	8,81594%	1,000	-31,0681%	28,4247%
		neurobasal a + vit c	9,91647%	8,81594%	1,000	-19,8299%	39,6629%
		neurobasal a + vit c + insulin	3,12632%	8,81594%	1,000	-26,6201%	32,8727%
	BME + vit c + insulin	dead ctrl	-1,12382%	8,24656%	1,000	-28,8776%	26,6299%
		medium ctrl	-25,57886%	8,81594%	0,170	-55,3252%	4,1675%
		BME + insulin	-18,85216%	9,24624%	0,764	-50,0181%	12,3138%
		neurobasal a	-20,48015%	8,49526%	0,466	-49,1232%	8,1629%
		BME + vit c	-16,61400%	8,81594%	0,867	-46,3604%	13,1324%
		neurobasal a + insulin	-17,93566%	8,81594%	0,769	-47,6820%	11,8107%
		neurobasal a + vit c	-6,69752%	8,81594%	1,000	-36,4439%	23,0489%
		neurobasal a + vit c + insulin	-13,48767%	8,81594%	0,984	-43,2341%	16,2587%
	neurobasal a + insulin	dead ctrl	16,81184%	8,24656%	0,762	-10,9419%	44,5656%
		medium ctrl	-7,64320%	8,81594%	1,000	-37,3896%	22,1032%
		BME + insulin	-0,91650%	9,24624%	1,000	-32,0825%	30,2495%
		neurobasal a	-2,54449%	8,49526%	1,000	-31,1876%	26,0986%
		BME + vit c	1,32166%	8,81594%	1,000	-28,4247%	31,0681%
		BME + vit c + insulin	17,93566%	8,81594%	0,769	-11,8107%	47,6820%
		neurobasal a + vit c	11,23814%	8,81594%	0,999	-18,5082%	40,9845%
		neurobasal a + vit c + insulin	4,44799%	8,81594%	1,000	-25,2984%	34,1944%
	neurobasal a + vit c	dead ctrl	5,57370%	8,24656%	1,000	-22,1801%	33,3275%
		medium ctrl	-18,88134%	8,81594%	0,686	-48,6277%	10,8650%
		BME + insulin	-12,15464%	9,24624%	0,998	-43,3206%	19,0113%
		neurobasal a	-13,78263%	8,49526%	0,968	-42,4257%	14,8605%
		BME + vit c	-9,91647%	8,81594%	1,000	-39,6629%	19,8299%
		BME + vit c + insulin	6,69752%	8,81594%	1,000	-23,0489%	36,4439%
		neurobasal a + insulin	-11,23814%	8,81594%	0,999	-40,9845%	18,5082%
		neurobasal a + vit c + insulin	-6,79015%	8,81594%	1,000	-36,5365%	22,9562%
	neurobasal a + vit c + insulin	dead ctrl	12,36385%	8,24656%	0,987	-15,3899%	40,1176%
		medium ctrl	-12,09119%	8,81594%	0,996	-41,8376%	17,6552%
		BME + insulin	-5,36449%	9,24624%	1,000	-36,5304%	25,8015%
		neurobasal a	-6,99248%	8,49526%	1,000	-35,6356%	21,6506%
		BME + vit c	-3,12632%	8,81594%	1,000	-32,8727%	26,6201%
		BME + vit c + insulin	13,48767%	8,81594%	0,984	-16,2587%	43,2341%
		neurobasal a + insulin	-4,44799%	8,81594%	1,000	-34,1944%	25,2984%
		neurobasal a + vit c	6,79015%	8,81594%	1,000	-22,9562%	36,5365%

Table 6: Cortex day 1 post-hoc test following significant ANOVA. Significant p-values are highlighted in green.

Post-hoc test

Abhängige Variable:

group			Mittelwertdifferenz (I-J)	Std.-Fehler	Sig.	95% Konfidenzintervall Untergrenze	95% Konfidenzintervall Obergrenze
Gabriel	BME + insulin	neurobasal a	-40,24547%*	10,39695%	0,012	-74,5969%	-5,8940%
		BME + vit c	-17,78192%	13,42241%	0,970	-61,1940%	25,6302%
		BME + vit c + insulin	-14,33765%	11,38929%	0,984	-51,8360%	23,1607%
		neurobasal a + insulin	-14,12065%	13,42241%	0,997	-57,5327%	29,2915%
		neurobasal a + vit c	-26,00008%	12,19151%	0,515	-65,8972%	13,8970%
		neurobasal a + vit c + insulin	-7,34394%	10,82149%	1,000	-43,0716%	28,3837%
	neurobasal a	BME + insulin	40,24547%*	10,39695%	0,012	5,8940%	74,5969%
		BME + vit c	22,46355%	13,42241%	0,830	-20,9486%	65,8756%
		BME + vit c + insulin	25,90782%	11,38929%	0,423	-11,5905%	63,4062%
		neurobasal a + insulin	26,12482%	13,42241%	0,638	-17,2873%	69,5369%
		neurobasal a + vit c	14,24539%	12,19151%	0,992	-25,6517%	54,1425%
		neurobasal a + vit c + insulin	32,90153%	10,82149%	0,093	-2,8261%	68,6292%
	BME + vit c	BME + insulin	17,78192%	13,42241%	0,970	-25,6302%	61,1940%
		neurobasal a	-22,46355%	13,42241%	0,830	-65,8756%	20,9486%
		BME + vit c + insulin	3,44427%	14,20494%	1,000	-43,1147%	50,0032%
		neurobasal a + insulin	3,66127%	15,88161%	1,000	-48,8115%	56,1340%
		neurobasal a + vit c	-8,21816%	14,85588%	1,000	-57,1759%	40,7396%
		neurobasal a + vit c + insulin	10,43798%	13,75387%	1,000	-34,3503%	55,2263%
	BME + vit c + insulin	BME + insulin	14,33765%	11,38929%	0,984	-23,1607%	51,8360%
		neurobasal a	-25,90782%	11,38929%	0,423	-63,4062%	11,5905%
		BME + vit c	-3,44427%	14,20494%	1,000	-50,0032%	43,1147%
		neurobasal a + insulin	0,21700%	14,20494%	1,000	-46,3420%	46,7760%
		neurobasal a + vit c	-11,66243%	13,04807%	1,000	-54,7064%	31,3815%
		neurobasal a + vit c + insulin	6,99371%	11,77811%	1,000	-31,8808%	45,8682%
	neurobasal a + insulin	BME + insulin	14,12065%	13,42241%	0,997	-29,2915%	57,5327%
		neurobasal a	-26,12482%	13,42241%	0,638	-69,5369%	17,2873%
		BME + vit c	-3,66127%	15,88161%	1,000	-56,1340%	48,8115%
		BME + vit c + insulin	-0,21700%	14,20494%	1,000	-46,7760%	46,3420%
		neurobasal a + vit c	-11,87943%	14,85588%	1,000	-60,8372%	37,0783%
		neurobasal a + vit c + insulin	6,77670%	13,75387%	1,000	-38,0116%	51,5650%
	neurobasal a + vit c	BME + insulin	26,00008%	12,19151%	0,515	-13,8970%	65,8972%
		neurobasal a	-14,24539%	12,19151%	0,992	-54,1425%	25,6517%
		BME + vit c	8,21816%	14,85588%	1,000	-40,7396%	57,1759%
		BME + vit c + insulin	11,66243%	13,04807%	1,000	-31,3815%	54,7064%
		neurobasal a + insulin	11,87943%	14,85588%	1,000	-37,0783%	60,8372%
		neurobasal a + vit c + insulin	18,65614%	12,55551%	0,933	-22,6171%	59,9294%
	neurobasal a + vit c + insulin	BME + insulin	7,34394%	10,82149%	1,000	-28,3837%	43,0716%
		neurobasal a	-32,90153%	10,82149%	0,093	-68,6292%	2,8261%
		BME + vit c	-10,43798%	13,75387%	1,000	-55,2263%	34,3503%
		BME + vit c + insulin	-6,99371%	11,77811%	1,000	-45,8682%	31,8808%
		neurobasal a + insulin	-6,77670%	13,75387%	1,000	-51,5650%	38,0116%
		neurobasal a + vit c	-18,65614%	12,55551%	0,933	-59,9294%	22,6171%

*. Die Mittelwertdifferenz ist in Stufe 0.05 signifikant.

Table 7: Cortex day 3 post-hoc test following significant ANOVA. Significant p-values are highlighted in green.

Paarweise Vergleiche von group

Sample 1-Sample 2	Teststatistik	Standardfehler	Standardteststatistik	Sig.	Anp. Sig. Neu[a]
dead ctrl-medium ctrl	-9,819	7,364	-1,333	0,182	2,736
dead ctrl-BME + insulin	-17,016	7,637	-2,228	0,026	0,388205933
dead ctrl-neurobasal a + vit c + insulin	-21,278	7,987	-2,664	0,008	0,115838351
dead ctrl-neurobasal a + insulin	-24,111	10,103	-2,386	0,017	0,255
dead ctrl-BME + vit c + insulin	-24,244	8,453	-2,868	0,004	0,061927265
dead ctrl-BME + vit c	-26,778	10,103	-2,650	0,008	0,120583052
dead ctrl-neurobasal a + vit c	-31,694	9,107	-3,480	0,001	0,008
dead ctrl-neurobasal a	-37,016	7,637	-4,847	0,000	1,88265E-05
medium ctrl-BME + insulin	-7,196	7,843	-0,918	0,359	5,38303866
medium ctrl-neurobasal a + vit c + insulin	-11,458	8,185	-1,400	0,162	2,423
medium ctrl-neurobasal a + insulin	-14,292	10,260	-1,393	0,164	2,45441243
medium ctrl-BME + vit c + insulin	-14,425	8,640	-1,670	0,095	1,424832718
medium ctrl-BME + vit c	-16,958	10,260	-1,653	0,098	1,475
medium ctrl-neurobasal a + vit c	-21,875	9,280	-2,357	0,018	0,276252341
medium ctrl-neurobasal a	-27,196	7,843	-3,467	0,001	0,007881072

Jede Zeile prüft die Nullhypothese, dass die Verteilungen in Stichprobe 1 und Stichprobe 2 gleich sind.
Asymptotische Signifikanzen (zweiseitige Tests) werden angezeigt. Das Signifikanzniveau ist ,050.
a. Signifikanzwerte wurden durch Bonferroni-Korrektur für mehrere Tests angepasst.

Table 8: Kruskal-Wallis' test for cortex day 3 with Bonferroni correction (15 comparisons). Significant, corrected p-values are highlighted in green.

Paarweise Vergleiche von group

Sample 1-Sample 2	Teststatistik	Standardfehler	Standardteststatistik	Sig.	Anp. Sig. Neu[a]
dead ctrl-medium ctrl	-12,014	7,504	-1,601	0,109	2,844014846
dead ctrl-BME + insulin	-16,032	7,783	-2,060	0,039	1,024601466
dead ctrl-neurobasal a + insulin	-19,889	16,279	-1,222	0,222	5,766711834
dead ctrl-BME + vit c + insulin	-21,489	8,614	-2,495	0,013	0,327799048
dead ctrl-neurobasal a + vit c + insulin	-24,889	8,614	-2,889	0,004	0,100362947
dead ctrl-neurobasal a	-25,556	7,280	-3,510	0,000	0,011636519
dead ctrl-neurobasal a + vit c	-26,889	10,296	-2,612	0,009	0,234256376
dead ctrl-BME + vit c	-29,556	8,139	-3,631	0,000	0,007335715
medium ctrl-BME + insulin	-4,018	7,993	-0,503	0,615	15,99477134
medium ctrl-neurobasal a + insulin	-7,875	16,380	-0,481	0,631	16,39782766
medium ctrl-BME + vit c + insulin	-9,475	8,804	-1,076	0,282	7,327772979
medium ctrl-neurobasal a + vit c + insulin	-12,875	8,804	-1,462	0,144	3,734537766
medium ctrl-neurobasal a	-13,542	7,504	-1,805	0,071	1,849759881
medium ctrl-neurobasal a + vit c	-14,875	10,455	-1,423	0,155	4,025186029
medium ctrl-BME + vit c	-17,542	8,340	-2,103	0,035	0,921625951
BME + insulin-neurobasal a + insulin	-3,857	16,510	-0,234	0,815	21,19711125
BME + insulin-BME + vit c + insulin	-5,457	9,043	-0,603	0,546	14,20088522
neurobasal a + insulin-BME + vit c + insulin	1,600	16,917	0,095	0,925	24,04092295
neurobasal a + insulin-neurobasal a + vit c + insulin	-5,000	16,917	-0,296	0,768	19,95686072
neurobasal a + insulin-neurobasal a	5,667	16,279	0,348	0,728	18,92188114
neurobasal a + insulin-neurobasal a + vit c	-7,000	17,833	-0,393	0,695	18,06113106
neurobasal a + insulin-BME + vit c	9,667	16,681	0,580	0,562	14,61841423
BME + vit c + insulin-neurobasal a + vit c + insulin	-3,400	9,767	-0,348	0,728	18,92188114
BME + vit c + insulin-neurobasal a	4,067	8,614	0,472	0,637	16,55818781
BME + vit c + insulin-neurobasal a + vit c	-5,400	11,278	-0,479	0,632	16,43418414
BME + vit c + insulin-BME + vit c	8,067	9,351	0,863	0,388	10,09715826

Jede Zeile prüft die Nullhypothese, dass die Verteilungen in Stichprobe 1 und Stichprobe 2 gleich sind.
Asymptotische Signifikanzen (zweiseitige Tests) werden angezeigt. Das Signifikanzniveau ist ,050.
a. Signifikanzwerte wurden durch Bonferroni-Korrektur für mehrere Tests angepasst.

Table 9: Kruskal-Wallis' test for hippocampus day 3 with Bonferroni correction (26 comparisons). Significant, corrected p-values are highlighted in green.

Post-hoc test

Abhängige Variable:

group			Mittelwertdifferenz (I-J)	Std.-Fehler	Sig.	95% Untergrenze	95% Obergrenze
Gabriel	dead ctrl	medium ctrl	-8,35741%	8,57259%	1,000	-37,0557%	20,3409%
		BME + insulin	-9,58112%	8,57259%	1,000	-38,2794%	19,1171%
		neurobasal a	-10,02912%	7,56031%	0,998	-35,6425%	15,5843%
		BME + vit c	-28,56873%*	7,98065%	0,028	-55,4922%	-1,6453%
		BME + vit c + insulin	-2,87464%	7,56031%	1,000	-28,4880%	22,7387%
		neurobasal a + insulin	-23,87074%	7,24516%	0,065	-48,4659%	0,7244%
		neurobasal a + vit c	-16,27518%	7,56031%	0,673	-41,8886%	9,3382%
		neurobasal a + vit c + insulin	-29,60916%*	7,98065%	0,019	-56,5326%	-2,6857%
	medium ctrl	dead ctrl	8,35741%	8,57259%	1,000	-20,3409%	37,0557%
		BME + insulin	-1,22371%	9,89878%	1,000	-34,8458%	32,3984%
		neurobasal a	-1,67170%	9,03630%	1,000	-32,2089%	28,8655%
		BME + vit c	-20,21132%	9,39080%	0,675	-52,0586%	11,6360%
		BME + vit c + insulin	5,48278%	9,03630%	1,000	-25,0544%	36,0200%
		neurobasal a + insulin	-15,51333%	8,77433%	0,914	-45,0324%	14,0057%
		neurobasal a + vit c	-7,91777%	9,03630%	1,000	-38,4550%	22,6195%
		neurobasal a + vit c + insulin	-21,25175%	9,39080%	0,585	-53,0991%	10,5956%
	BME + insulin	dead ctrl	9,58112%	8,57259%	1,000	-19,1171%	38,2794%
		medium ctrl	1,22371%	9,89878%	1,000	-32,3984%	34,8458%
		neurobasal a	-0,44799%	9,03630%	1,000	-30,9852%	30,0892%
		BME + vit c	-18,98761%	9,39080%	0,774	-50,8349%	12,8597%
		BME + vit c + insulin	6,70649%	9,03630%	1,000	-23,8307%	37,2437%
		neurobasal a + insulin	-14,28962%	8,77433%	0,961	-43,8086%	15,2294%
		neurobasal a + vit c	-6,69406%	9,03630%	1,000	-37,2313%	23,8432%
		neurobasal a + vit c + insulin	-20,02804%	9,39080%	0,690	-51,8754%	11,8193%
	neurobasal a	dead ctrl	10,02912%	7,56031%	0,998	-15,5843%	35,6425%
		medium ctrl	1,67170%	9,03630%	1,000	-28,8655%	32,2089%
		BME + insulin	0,44799%	9,03630%	1,000	-30,0892%	30,9852%
		BME + vit c	-18,53962%	8,47681%	0,648	-47,3021%	10,2228%
		BME + vit c + insulin	7,15448%	8,08232%	1,000	-20,2979%	34,6068%
		neurobasal a + insulin	-13,84163%	7,78832%	0,917	-40,2758%	12,5925%
		neurobasal a + vit c	-6,24607%	8,08232%	1,000	-33,6984%	21,2063%
		neurobasal a + vit c + insulin	-19,58004%	8,47681%	0,548	-48,3425%	9,1824%
	BME + vit c	dead ctrl	28,56873%*	7,98065%	0,028	1,6453%	55,4922%
		medium ctrl	20,21132%	9,39080%	0,675	-11,6360%	52,0586%
		BME + insulin	18,98761%	9,39080%	0,774	-12,8597%	50,8349%
		neurobasal a	18,53962%	8,47681%	0,648	-10,2228%	47,3021%
		BME + vit c + insulin	25,69410%	8,47681%	0,128	-3,0683%	54,4565%
		neurobasal a + insulin	4,69799%	8,19697%	1,000	-23,0462%	32,4422%
		neurobasal a + vit c	12,29355%	8,47681%	0,991	-16,4689%	41,0560%
		neurobasal a + vit c + insulin	-1,04043%	8,85373%	1,000	-31,1130%	29,0321%
	BME + vit c + insulin	dead ctrl	2,87464%	7,56031%	1,000	-22,7387%	28,4880%
		medium ctrl	-5,48278%	9,03630%	1,000	-36,0200%	25,0544%
		BME + insulin	-6,70649%	9,03630%	1,000	-37,2437%	23,8307%
		neurobasal a	-7,15448%	8,08232%	1,000	-34,6068%	20,2979%
		BME + vit c	-25,69410%	8,47681%	0,128	-54,4565%	3,0683%
		neurobasal a + insulin	-20,99611%	7,78832%	0,274	-47,4302%	5,4380%
		neurobasal a + vit c	-13,40055%	8,08232%	0,958	-40,8529%	14,0518%
		neurobasal a + vit c + insulin	-26,73452%	8,47681%	0,094	-55,4970%	2,0279%
	neurobasal a + insulin	dead ctrl	23,87074%	7,24516%	0,065	-0,7244%	48,4659%
		medium ctrl	15,51333%	8,77433%	0,914	-14,0057%	45,0324%
		BME + insulin	14,28962%	8,77433%	0,961	-15,2294%	43,8086%
		neurobasal a	13,84163%	7,78832%	0,917	-12,5925%	40,2758%
		BME + vit c	-4,69799%	8,19697%	1,000	-32,4422%	23,0462%
		BME + vit c + insulin	20,99611%	7,78832%	0,274	-5,4380%	47,4302%
		neurobasal a + vit c	7,59556%	7,78832%	1,000	-18,8386%	34,0297%
		neurobasal a + vit c + insulin	-5,73842%	8,19697%	1,000	-33,4826%	22,0058%
	neurobasal a + vit c	dead ctrl	16,27518%	7,56031%	0,673	-9,3382%	41,8886%
		medium ctrl	7,91777%	9,03630%	1,000	-22,6195%	38,4550%
		BME + insulin	6,69406%	9,03630%	1,000	-23,8432%	37,2313%
		neurobasal a	6,24607%	8,08232%	1,000	-21,2063%	33,6984%
		BME + vit c	-12,29355%	8,47681%	0,991	-41,0560%	16,4689%
		BME + vit c + insulin	13,40055%	8,08232%	0,958	-14,0518%	40,8529%
		neurobasal a + insulin	-7,59556%	7,78832%	1,000	-34,0297%	18,8386%
		neurobasal a + vit c + insulin	-13,33398%	8,47681%	0,976	-42,0964%	15,4285%
	neurobasal a + vit c + insulin	dead ctrl	29,60916%*	7,98065%	0,019	2,6857%	56,5326%
		medium ctrl	21,25175%	9,39080%	0,585	-10,5956%	53,0991%
		BME + insulin	20,02804%	9,39080%	0,690	-11,8193%	51,8754%
		neurobasal a	19,58004%	8,47681%	0,548	-9,1824%	48,3425%
		BME + vit c	1,04043%	8,85373%	1,000	-29,0321%	31,1130%
		BME + vit c + insulin	26,73452%	8,47681%	0,094	-2,0279%	55,4970%
		neurobasal a + insulin	5,73842%	8,19697%	1,000	-22,0058%	33,4826%
		neurobasal a + vit c	13,33398%	8,47681%	0,976	-15,4285%	42,0964%

*. Die Mittelwertdifferenz ist in Stufe 0.05 signifikant.

Table 10: Thalamus day 1 post-hoc test following significant ANOVA. Significant p-values are highlighted in green.

Paarweise Vergleiche von group

Sample 1-Sample 2	Teststatistik	Standardfehler	Standardteststatistik	Sig.	Anp. Sig. Neu [a]
dead ctrl-BME + insulin	-11,952	7,637	-1,565	0,118	1,76372609
dead ctrl-medium ctrl	-17,042	7,364	-2,314	0,021	0,309833864
dead ctrl-neurobasal a + vit c + insulin	-22,667	8,453	-2,682	0,007	0,109933058
dead ctrl-BME + vit c	-25,667	9,107	-2,818	0,005	0,072399223
dead ctrl-neurobasal a + vit c	-25,917	9,107	-2,846	0,004	0,066442687
dead ctrl-BME + vit c + insulin	-29,952	7,637	-3,922	0,000	0,001317993
dead ctrl-neurobasal a + insulin	-30,000	10,103	-2,969	0,003	0,04476244
dead ctrl-neurobasal a	-31,467	8,453	-3,723	0,000	0,002957965
BME + insulin-medium ctrl	5,089	7,843	0,649	0,516	7,746357286
BME + insulin-neurobasal a + vit c + insulin	-10,714	8,874	-1,207	0,227	3,409068217
BME + insulin-BME + vit c	-13,714	9,499	-1,444	0,149	2,231930804
BME + insulin-neurobasal a + vit c	-13,964	9,499	-1,470	0,142	2,12294394
BME + insulin-BME + vit c + insulin	-18,000	8,101	-2,222	0,026	0,394179627
BME + insulin-neurobasal a + insulin	-18,048	10,458	-1,726	0,084	1,265857046
BME + insulin-neurobasal a	-19,514	8,874	-2,199	0,028	0,418050869

Jede Zeile prüft die Nullhypothese, dass die Verteilungen in Stichprobe 1 und Stichprobe 2 gleich sind.
Asymptotische Signifikanzen (zweiseitige Tests) werden angezeigt. Das Signifikanzniveau ist ,050.
a. Signifikanzwerte wurden durch Bonferroni-Korrektur für mehrere Tests angepasst.

Table 11: Kruskal-Wallis' test for thalamus day 3 with Bonferroni correction (15 comparisons). Significant, corrected p-values are highlighted in green.

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