Aus der Klinik für Neurologie mit Experimenteller Neurologie
der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

Neuronal culture microenvironments determine preferences in bioenergetic pathway use

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Doctor of Philosophy (PhD)

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Abstrakt in Deutsch

Im Gehirn sind die metabolische Versorgung und der metabolische Bedarf direkt mit der neuronalen Aktivität verknüpft. Methoden zur Kultivierung primärer Nagetierhirnzellen sind ausgereift und richten sich auf die Modellierung der humanen Gehirnphysiologie und Pathologie. Dennoch wurde die Bedeutung der Kultivierungsbedingungen auf die neuronale Funktion bisher kaum betrachtet.

Aus diesem Grund haben wir die Rolle der verschiedenen Kultivierungszusätze auf das neuronale Überleben und die metabolische Aktivität in einem metabolischen Entzugsmodell untersucht. Dieses beinhaltet den Entzug von Sauerstoff bzw. Glukose sowie die Messung des metabolischen Flusses an lebenden Zellen.

Wir konnten die Bedeutung der neuronalen Kultivierungsbedingungen auf die metabolische Funktion und das neuronale Überleben unter metabolischen Stressbedingungen demonstrieren. Insbesondere haben wir herausgefunden, dass B27, ein verbreiteter neuronaler Kultivierungszusatz, Neurone vor dem Zelltod unter hypoxischen Bedingungen schützt und die Glykolyse inhibiert. Außerdem konnten wir zeigen, dass B27, genau wie der alternative Kultivierungszusatz N2, den neuronalen Glukosemetabolismus einschränkt. Im Gegensatz dazu haben wir herausgefunden, dass der modernere Zusatz GS21 den neuronalen Energiemetabolismus unterstützt.

Unsere Daten stützen die Aussage, dass die sorgfältige Kontrolle der metabolischen Kultivierungsbedingungen eine essentielle Komponente bei der Modellierung der Hirnfunktion, der zellulären und der molekularen Pathophysiologie von Gehirnerkrankungen in der Zellkultur darstellt.

Abstract in English

In the brain, metabolic supply and demand is directly coupled to neuronal activation. Methods for culturing primary rodent brain cells have come of age and are geared toward sophisticated modeling of human brain physiology and pathology. However, the impact of the culture microenvironment on neuronal function is rarely considered.

Therefore, we investigated the role of different neuronal culture supplements for neuronal survival and metabolic activity in a model of metabolic deprivation of neurons using oxygen deprivation, glucose deprivation, as well as live cell metabolic flux analysis.

We demonstrate the impact of neuronal culture conditions on metabolic function and neuronal survival under conditions of metabolic stress. In particular we find that the common neuronal cell culture supplement B27 protects neurons from cell death under hypoxic conditions and inhibits glycolysis. Furthermore, we present data that B27 as well as the alternative neuronal culture supplement N2 restrict neuronal glucose metabolism. On the contrary, we find that the more modern supplement GS21 promotes neuronal energy metabolism.

Our data support the notion that careful control of the metabolic environment is an essential component in modeling brain function and the cellular and molecular pathophysiology of brain disease in culture.

Eidesstattliche Versicherung

„Ich, Juliane Sünwoldt, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: „Neuronal culture microenvironments determine preferences in bioenergetic pathway use“ selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autoren beruhen, sind als solche in korrekter Zitierung (siehe „Uniform Requirements for Manuscripts (URM)“ des ICMJE -*www.**icmje**.org*) kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen) entsprechen den URM (s. o.) und werden von mir verantwortet.

Mein Anteil an der ausgewählten Publikation entspricht dem, der in der untenstehenden gemeinsamen Erklärung mit dem Betreuer, angegeben ist. Sämtliche Publikationen, die aus dieser Dissertation hervorgegangen sind und bei denen ich Autor bin, entsprechen den URM (s. o.) und werden von mir verantwortet.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§§156,161 des Strafgesetzbuches) sind mir bekannt und bewusst.“

11.12.2017 Unterschrift

Ausführliche Anteilserklärung an der erfolgten Publikation

Publikation: Sünwoldt, J., Bosche, B., Meisel, A., Mergenthaler, P. Neuronal culture microenvironments determine preferences in bioenergetic pathway use. Frontiers in Molecular Neuroscience. 2017. 10, 305. doi: 10.3389/fnmol.2017.00305.

Beitrag im Einzelnen:

1. Planung und Organisation aller Experimente in Absprache mit Herrn Dr. Mergenthaler.
2. Durchführung aller Experimente, im Einzelnen: Präparation der primären Cortexneurone, Aussaat und Kultivierung der Zellen, Messung der metabolischen Parameter zur Bestimmung der Glykolyse und der oxidativen Phosphorylierung mittels Seahorse XFe96 Extracellular Flux Analyzer, Messung des Laktatgehalts im Zellmedium, Bestimmung der Freisetzung der Laktatdehydrogenase nach der Durchführung von Deprivationsexperimenten.
3. Aufbereitung und Auswertung aller Daten sowie Durchführung der statistischen Analysen.
4. Interpretation der Ergebnisse, Erstellung der Abbildungen und Tabellen, Verfassen des ersten Paper-Entwurfs und Mitarbeit an der finalen Version des Papers.

Unterschrift der Doktorandin

Auszug aus der Journal Summary List (ISI Web of Knowledge^{SM})

Die Publikation „Neuronal culture microenvironments determine preferences in bioenergetic pathway use” wurde am 29.09.2017 in Frontiers of Molecular Neuroscience publiziert. Die Fachzeitschrift liegt auf Rang 42 von 258 im Bereich der Neurowissenschaften mit einem Impact Faktor von 5,076 und einem Eigenfaktor von 0,008520. Im Folgenden befindet sich ein Auszug aus der Journal Summary List.

Journal Data Filtered By: **Selected JCR Year: 2016** Selected Editions: SCIE,SSCI
Selected Categories: **“NEUROSCIENCES”** Selected Category Scheme: WoS
Gesamtanzahl: 258 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	NATURE REVIEWS NEUROSCIENCE	36,952	28.880	0.071380
2	NATURE NEUROSCIENCE	54,399	17.839	0.160740
3	Annual Review of Neuroscience	13,211	15.630	0.020660
4	TRENDS IN COGNITIVE SCIENCES	23,273	15.402	0.046360
5	BEHAVIORAL AND BRAIN SCIENCES	8,195	14.200	0.010940
6	NEURON	82,253	14.024	0.227070
7	PROGRESS IN NEUROBIOLOGY	12,163	13.217	0.018020
8	MOLECULAR PSYCHIATRY	17,452	13.204	0.049670
9	ACTA NEUROPATHOLOGICA	16,462	12.213	0.037060
10	BIOLOGICAL PSYCHIATRY	41,859	11.412	0.067400
11	TRENDS IN NEUROSCIENCES	19,178	11.124	0.029690
12	JOURNAL OF PINEAL RESEARCH	7,278	10.391	0.008040
13	BRAIN	48,061	10.292	0.077590
14	ANNALS OF NEUROLOGY	34,215	9.890	0.057310
15	FRONTIERS IN NEUROENDOCRINOLOGY	3,516	9.425	0.006600
16	SLEEP MEDICINE REVIEWS	4,980	8.958	0.009730
17	NEUROSCIENCE AND BIOBEHAVIORAL REVIEWS	20,452	8.299	0.047230
18	NEUROSCIENTIST	4,325	7.391	0.009890
19	Molecular Neurodegeneration	2,946	6.780	0.009540
20	CEREBRAL CORTEX	27,496	6.559	0.063240
21	NEUROPSYCHOPHARMACOLOGY	23,920	6.403	0.046670
22	NEUROPSYCHOLOGY REVIEW	2,478	6.352	0.004650
23	GLIA	12,781	6.200	0.021920
24	Alzheimers Research & Therapy	1,699	6.196	0.007180
25	MOLECULAR NEUROBIOLOGY	7,338	6.190	0.017440
26	NEUROSIGNALS	653	6.143	0.000670
27	CURRENT OPINION IN NEUROBIOLOGY	13,188	6.133	0.036730
28	Brain Stimulation	3,905	6.078	0.013020
29	JOURNAL OF NEUROSCIENCE	171,800	5.988	0.319910
30	BRAIN BEHAVIOR AND IMMUNITY	10,719	5.964	0.026460
31	NEUROIMAGE	85,630	5.835	0.173210
32	PAIN	35,333	5.445	0.044460
33	NEUROPATHOLOGY AND APPLIED NEUROBIOLOGY	3,413	5.347	0.006400
34	NEURAL NETWORKS	8,741	5.287	0.010250
35	BRAIN PATHOLOGY	4,580	5.272	0.008450
36	JOURNAL OF NEUROTRAUMA	12,787	5.190	0.021640
37	Neurotherapeutics	3,451	5.166	0.008220
38	JOURNAL OF PSYCHIATRY & NEUROSCIENCE	2,759	5.165	0.004970
39	NEUROBIOLOGY OF AGING	20,010	5.117	0.046250

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
40	Journal of Neuroinflammation	7,946	5.102	0.023970
41	JOURNAL OF CEREBRAL BLOOD FLOW AND METABOLISM	16,998	5.081	0.029520
42	Frontiers in Molecular Neuroscience	1,979	5.076	0.008520
43	NEUROBIOLOGY OF DISEASE	14,554	5.020	0.031140
44	NEUROPHARMACOLOGY	18,559	5.012	0.040280
45	SLEEP	18,127	4.923	0.026090
46	Multiple Sclerosis Journal	9,727	4.840	0.023240
47	Molecular Autism	1,294	4.833	0.006320
48	PSYCHONEUROENDOCRINOLOGY	14,409	4.788	0.028830
49	Neuropsychiatry	149	4.778	0.000740
50	JOURNAL OF PHYSIOLOGY-LONDON	48,567	4.739	0.047830
51	INTERNATIONAL JOURNAL OF NEUROPSYCHOPHARMACOLOGY	6,082	4.712	0.015310
52	EXPERIMENTAL NEUROLOGY	19,445	4.706	0.027440
53	CURRENT OPINION IN NEUROLOGY	5,258	4.699	0.011490
54	Brain Structure & Function	4,325	4.698	0.014300
55	Frontiers in Cellular Neuroscience	6,088	4.555	0.027500
56	BIPOLAR DISORDERS	5,323	4.531	0.009660
57	HUMAN BRAIN MAPPING	18,139	4.530	0.041900
58	JOURNAL OF PAIN	8,312	4.519	0.018540
59	Frontiers in Aging Neuroscience	3,477	4.504	0.013020
60	Developmental Cognitive Neuroscience	1,483	4.321	0.007490
61	CORTEX	8,200	4.279	0.021370
62	EUROPEAN NEUROPSYCHOPHARMACOLOGY	6,575	4.239	0.015920
63	PROGRESS IN NEUROPSYCHOPHARMACOLOGY & BIOLOGICAL PSYCHIATRY	9,740	4.187	0.016310
64	JOURNAL OF PSYCHOPHARMACOLOGY	5,518	4.179	0.012020
65	JOURNAL OF NEUROCHEMISTRY	35,279	4.083	0.030170
66	EUROPEAN JOURNAL OF NEUROLOGY	9,137	3.988	0.018850
67	Dialogues in Clinical Neuroscience	2,348	3.976	0.005480
68	HIPPOCAMPUS	8,694	3.945	0.016170
69	Social Cognitive and Affective Neuroscience	5,263	3.937	0.020160
70	CNS Neuroscience & Therapeutics	2,615	3.919	0.007370
71	Annals of Clinical and Translational Neurology	902	3.901	0.004880
72	ACS Chemical Neuroscience	3,084	3.883	0.011020
73	Frontiers in Neuroinformatics	1,377	3.870	0.006310
74	CLINICAL NEUROPHYSIOLOGY	17,871	3.866	0.021920
75	NUTRITIONAL NEUROSCIENCE	1,192	3.765	0.001900
76	GENES BRAIN AND BEHAVIOR	3,385	3.743	0.006820
77	JOURNAL OF ALZHEIMERS DISEASE	14,542	3.731	0.036370

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
78	NEUROGASTROENTEROLOGY AND MOTILITY	6,608	3.617	0.016200
79	CEPHALALGIA	7,932	3.609	0.011650
80	NEUROENDOCRINOLOGY	4,525	3.608	0.005060
81	Journal of Neurodevelopmental Disorders	825	3.582	0.003040
82	JOURNAL OF HEADACHE AND PAIN	2,141	3.580	0.004980
83	Frontiers in Neuroscience	6,489	3.566	0.027070
84	Frontiers in Neurology	3,192	3.552	0.014480
85	NEUROBIOLOGY OF LEARNING AND MEMORY	5,862	3.543	0.012320
86	Molecular Pain	2,975	3.533	0.007370
87	Journal of NeuroEngineering and Rehabilitation	3,323	3.516	0.007320
88	JOURNAL OF NEUROPATHOLOGY AND EXPERIMENTAL NEUROLOGY	8,483	3.503	0.009270
89	JOURNAL OF NEUROENDOCRINOLOGY	5,524	3.470	0.007680
90	Journal of Neural Engineering	4,693	3.465	0.011570
91	Cognitive Computation	795	3.441	0.001670
92	Molecular Brain	1,778	3.410	0.006030
93	BRAIN TOPOGRAPHY	2,155	3.394	0.004500
94	Current Neuropharmacology	2,087	3.365	0.003690
95	Current Neurology and Neuroscience Reports	2,294	3.345	0.006630
96	Journal of Neuroimmune Pharmacology	2,199	3.339	0.005540
97	PSYCHOPHARMACOLOGY	23,655	3.308	0.030960
98	NEUROMOLECULAR MEDICINE	1,641	3.287	0.002890
99	NEUROPSYCHOLOGY	5,422	3.286	0.007930
100	NEUROSCIENCE	44,046	3.277	0.060260
101	Frontiers in Neuroanatomy	1,975	3.267	0.009260
102	JOURNAL OF COMPARATIVE NEUROLOGY	29,871	3.266	0.018330
103	COGNITIVE AFFECTIVE & BEHAVIORAL NEUROSCIENCE	3,303	3.263	0.007260
104	NEUROCHEMISTRY INTERNATIONAL	7,819	3.262	0.011400
105	JOURNAL OF SLEEP RESEARCH	4,390	3.259	0.006910
106	CEREBELLUM	2,155	3.234	0.005740
107	Frontiers in Human Neuroscience	12,836	3.209	0.056590
108	JOURNAL OF NEUROVIROLOGY	2,487	3.206	0.004340
109	NEUROINFORMATICS	1,043	3.200	0.003960
110	NEUROPSYCHOLOGIA	23,509	3.197	0.034950
111	JOURNAL OF COGNITIVE NEUROSCIENCE	16,713	3.108	0.027250
112	Frontiers in Behavioral Neuroscience	4,319	3.104	0.018660
113	NEUROTOXICOLOGY	6,005	3.100	0.007880
114	MOLECULAR AND CELLULAR NEUROSCIENCE	6,529	3.084	0.009130
115	NEURAL PLASTICITY	2,131	3.054	0.006420

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
116	BRAIN RESEARCH BULLETIN	8,761	3.033	0.007430
116	DEVELOPMENTAL NEUROSCIENCE	2,028	3.033	0.002940
118	ASN Neuro	781	3.030	0.002470
119	Purinergic Signalling	1,494	3.022	0.003400
120	EUROPEAN JOURNAL OF PAIN	6,221	3.019	0.011280
121	Frontiers in Neural Circuits	2,117	3.005	0.012100
122	BEHAVIOURAL BRAIN RESEARCH	23,285	3.002	0.040590
123	Developmental Neurobiology	2,624	2.972	0.008870
124	NEUROMUSCULAR DISORDERS	4,283	2.969	0.008960
125	Current Alzheimer Research	3,244	2.952	0.007700
126	NEUROTOXICITY RESEARCH	2,357	2.942	0.003690
127	EUROPEAN JOURNAL OF NEUROSCIENCE	26,124	2.941	0.032090
128	CELLULAR AND MOLECULAR NEUROBIOLOGY	3,642	2.939	0.005390
129	HEARING RESEARCH	8,833	2.906	0.011270
130	LEARNING & MEMORY	5,835	2.894	0.008950
131	Neurodegenerative Diseases	1,509	2.842	0.003880
132	PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR	12,453	2.748	0.011780
133	BRAIN RESEARCH	54,957	2.746	0.041790
134	Neurophotonics	243	2.740	0.001220
135	JOURNAL OF NEUROIMMUNOLOGY	9,420	2.720	0.010610
136	JOURNAL OF PHYSIOLOGY-PARIS	1,629	2.704	0.002430
137	Progress in Brain Research	7,582	2.680	0.008720
138	NEUROIMMUNOMODULATION	1,407	2.674	0.002020
139	PSYCHOPHYSIOLOGY	12,232	2.668	0.013530
140	NEUROLOGIC CLINICS	1,845	2.648	0.002980
141	Neuroscience Bulletin	1,204	2.624	0.003630
142	MUSCLE & NERVE	10,897	2.605	0.015990
143	STRESS-THE INT. JOURNAL ON THE BIOLOGY OF STRESS	2,051	2.590	0.004600
144	INTERNATIONAL JOURNAL OF PSYCHOPHYSIOLOGY	6,949	2.582	0.011480
145	NEUROCHEMICAL RESEARCH	8,300	2.581	0.011620
146	JOURNAL OF NEUROSCIENCE METHODS	14,651	2.554	0.017570
147	REVIEWS IN THE NEUROSCIENCES	1,572	2.546	0.003310
148	Journal of Parkinsons Disease	956	2.538	0.004240
149	RESTORATIVE NEUROLOGY AND NEUROSCIENCE	1,935	2.526	0.003460
150	Annual Review of Vision Science	59	2.522	0.000480
151	CHEMICAL SENSES	4,296	2.520	0.004140
152	CNS & Neurological Disorders-Drug Targets	2,447	2.506	0.005490
153	Frontiers in Neurorobotics	270	2.486	0.000750
153	NEUROPEPTIDES	1,873	2.486	0.002620

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
155	JOURNAL OF NEUROSCIENCE RESEARCH	12,794	2.481	0.009270
156	JARO-JOURNAL OF THE ASSOCIATION FOR RESEARCH IN OTOLARYNGOLOGY	2,000	2.455	0.004750
157	BEHAVIORAL NEUROSCIENCE	7,020	2.453	0.006230
158	SEIZURE-EUROPEAN JOURNAL OF EPILEPSY	4,289	2.448	0.008550
159	BRAIN AND LANGUAGE	6,186	2.439	0.009710
160	BRAIN AND COGNITION	6,424	2.432	0.008550
161	JOURNAL OF COMPARATIVE PHYSIOLOGY A-NEUROETHOLOGY SENSORY NEURAL AND BEHAVIORAL PHYSIOLOGY	5,156	2.429	0.005050
162	NEUROTOXICOLOGY AND TERATOLOGY	3,483	2.410	0.004070
163	JOURNAL OF NEUROPHYSIOLOGY	41,502	2.396	0.046140
164	JOURNAL OF NEURAL TRANSMISSION	6,198	2.392	0.008630
165	JOURNAL OF THE PERIPHERAL NERVOUS SYSTEM	1,484	2.361	0.002830
166	GAIT & POSTURE	11,367	2.347	0.016780
167	BMC NEUROSCIENCE	4,368	2.312	0.008820
168	CURRENT NEUROVASCULAR RESEARCH	907	2.298	0.001440
169	METABOLIC BRAIN DISEASE	2,033	2.297	0.003860
170	JOURNAL OF THE NEUROLOGICAL SCIENCES	15,454	2.295	0.021780
171	JOURNAL OF NEUROGENETICS	590	2.291	0.001490
172	NEUROPSYCHOLOGICAL REHABILITATION	1,662	2.280	0.002760
173	Social Neuroscience	1,427	2.255	0.003850
174	JOURNAL OF MOLECULAR NEUROSCIENCE	4,746	2.229	0.010260
175	AUTONOMIC NEUROSCIENCE-BASIC & CLINICAL	2,488	2.225	0.004430
176	BEHAVIOURAL PHARMACOLOGY	2,748	2.218	0.003490
177	Behavioral and Brain Functions	1,533	2.207	0.002370
178	JOURNAL OF THE INTERNATIONAL NEUROPSYCHOLOGICAL SOCIETY	5,824	2.181	0.008680
179	NEUROSCIENCE LETTERS	32,352	2.180	0.035070
180	CLINICAL EEG AND NEUROSCIENCE	869	2.163	0.001890
181	Brain and Behavior	919	2.157	0.004090
182	SYNAPSE	4,361	2.132	0.004260
183	Neural Development	907	2.077	0.002730
184	PSYCHIATRY AND CLINICAL NEUROSCIENCES	2,856	2.063	0.003630
185	NEUROSCIENCE RESEARCH	4,692	2.060	0.006060

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
186	INTERNATIONAL JOURNAL OF DEVELOPMENTAL NEUROSCIENCE	3,152	2.046	0.005030
187	Clinical Psychopharmacology and Neuroscience	329	2.000	0.001110
188	BRAIN INJURY	5,470	1.971	0.008430
188	VISION RESEARCH	15,982	1.971	0.013590
190	ACTA NEUROPSYCHIATRICA	570	1.939	0.001330
191	NEURAL COMPUTATION	9,746	1.938	0.006660
192	JOURNAL OF CHEMICAL NEUROANATOMY	2,008	1.925	0.002190
193	EXPERIMENTAL BRAIN RESEARCH	20,713	1.917	0.018910
194	BRAIN BEHAVIOR AND EVOLUTION	2,100	1.915	0.002280
195	Cognitive Neuroscience	390	1.870	0.001520
196	JOURNAL OF NEUROPSYCHIATRY AND CLINICAL NEUROSCIENCES	3,431	1.846	0.003320
197	HUMAN MOVEMENT SCIENCE	3,776	1.841	0.005900
198	Cognitive Neurodynamics	603	1.828	0.001140
199	Frontiers in Computational Neuroscience	1,714	1.821	0.008070
200	AUDIOLOGY AND NEURO-OTOLOGY	1,659	1.791	0.002780
201	NEUROPATHOLOGY	1,547	1.784	0.002910
202	Neural Regeneration Research	1,886	1.769	0.005290
203	NEUROLOGY INDIA	1,897	1.758	0.002200
204	INTERNATIONAL JOURNAL OF NEUROSCIENCE	2,967	1.750	0.003370
205	NEUROLOGICAL SCIENCES	3,949	1.749	0.008950
206	VISUAL NEUROSCIENCE	2,446	1.737	0.002520
207	ACTA NEUROLOGICA BELGICA	741	1.722	0.001380
208	BIOLOGICAL CYBERNETICS	4,499	1.716	0.002420
209	EUROPEAN NEUROLOGY	2,952	1.697	0.003420
210	STEREOTACTIC AND FUNCTIONAL NEUROSURGERY	1,412	1.692	0.001890
211	IEEE Transactions on Autonomous Mental Development	347	1.638	0.000680
212	NEUROPHYSIOLOGIE CLINIQUE-CLINICAL NEUROPHYSIOLOGY	1,116	1.593	0.001270
213	JOURNAL OF CLINICAL NEUROSCIENCE	6,485	1.557	0.013610
213	PSYCHIATRIC GENETICS	1,002	1.557	0.001600
215	Journal of Stroke & Cerebrovascular Diseases	3,864	1.517	0.012700
216	JOURNAL OF ELECTROMYOGRAPHY AND KINESIOLOGY	4,667	1.510	0.006220
217	NEUROPSYCHOBIOLOGY	2,540	1.491	0.002560
218	JOURNAL OF MUSCULOSKELETAL & NEURONAL INTERACTIONS	1,439	1.489	0.001970
219	JOURNAL OF COMPUTATIONAL NEUROSCIENCE	1,814	1.483	0.003420
220	Chemosensory Perception	317	1.474	0.000660
221	JOURNAL OF NEUROLINGUISTICS	976	1.403	0.001760

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
222	NEUROREPORT	13,876	1.395	0.006850
223	NEUROLOGICAL RESEARCH	3,415	1.376	0.004420
224	ACTAS ESPANOLAS DE PSIQUIATRIA	548	1.339	0.000690
225	JOURNAL OF MOTOR BEHAVIOR	1,955	1.327	0.001760
226	NEUROIMAGING CLINICS OF NORTH AMERICA	1,017	1.325	0.001350
227	CLINICAL AUTONOMIC RESEARCH	1,333	1.276	0.001630
228	JOURNAL OF CLINICAL NEUROPHYSIOLOGY	2,866	1.224	0.003190
229	Computational Intelligence and Neuroscience	1,320	1.215	0.005020
230	ACTA NEUROBIOLOGIAE EXPERIMENTALIS	1,173	1.207	0.001520
231	Cognitive Systems Research	492	1.182	0.000730
232	FOLIA NEUROPATHOLOGICA	611	1.093	0.000830
233	INVERTEBRATE NEUROSCIENCE	286	0.947	0.000280
234	Sleep and Biological Rhythms	436	0.926	0.000880
235	Translational Neuroscience	220	0.922	0.000830
236	NEUROENDOCRINOLOGY LETTERS	1,955	0.918	0.002300
237	SOMATOSENSORY AND MOTOR RESEARCH	823	0.909	0.000480
238	ARQUIVOS DE NEURO-PSIQUIATRIA	2,562	0.902	0.003310
239	JOURNAL OF VESTIBULAR RESEARCH-EQUILIBRIUM & ORIENTATION	816	0.900	0.001050
240	ACUPUNCTURE & ELECTRO-THERAPEUTICS RESEARCH	183	0.870	0.000080
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242	MOTOR CONTROL	637	0.750	0.000720
243	ENCEPHALE-REVUE DE PSYCHIATRIE CLINIQUE BIOLOGIQUE ET THERAPEUTIQUE	1,171	0.742	0.001000
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248	NeuroQuantology	259	0.586	0.000300
249	ARCHIVES ITALIENNES DE BIOLOGIE	581	0.580	0.001050
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257	Journal of Neurological Sciences-Turkish	95	0.123	0.000190
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Neuronal Culture Microenvironments Determine Preferences in Bioenergetic Pathway Use

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In the brain, metabolic supply and demand is directly coupled to neuronal activation. Methods for culturing primary rodent brain cells have come of age and are geared toward sophisticated modeling of human brain physiology and pathology. However, the impact of the culture microenvironment on neuronal function is rarely considered. Therefore, we investigated the role of different neuronal culture supplements for neuronal survival and metabolic activity in a model of metabolic deprivation of neurons using oxygen deprivation, glucose deprivation, as well as live cell metabolic flux analysis. We demonstrate the impact of neuronal culture conditions on metabolic function and neuronal survival under conditions of metabolic stress. In particular, we find that the common neuronal cell culture supplement B27 protects neurons from cell death under hypoxic conditions and inhibits glycolysis. Furthermore, we present data that B27 as well as the alternative neuronal culture supplement N2 restrict neuronal glucose metabolism. On the contrary, we find that the more modern supplement GS21 promotes neuronal energy metabolism. Our data support the notion that careful control of the metabolic environment is an essential component in modeling brain function and the cellular and molecular pathophysiology of brain disease in culture.

Keywords: disease modeling, energy metabolism, glycolysis, metabolic flux analysis, neuronal energy metabolism, neuronal survival, oxidative phosphorylation, cell culture microenvironment

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INTRODUCTION

In the brain, neuronal function is directly coupled to metabolic activity, and neuronal computation regulates metabolic supply and demand (Dohmen et al., 2003; Bosche et al., 2010; Mergenthaler et al., 2013; Magistretti and Allaman, 2015). Indeed, the brain uses most of its energy consumption to sustain synaptic activity under physiological conditions (Alle et al., 2009; Harris et al., 2012; Mergenthaler et al., 2013). In addition to a central role for the pathophysiology of epileptic seizures (Arsov et al., 2012; Lutas and Yellen, 2013) or cortical spreading depressions (Dohmen et al., 2008; Bosche et al., 2010; Feuerstein et al., 2016), glucose metabolism, metabolic deprivation, and disturbed metabolic pathways are emerging as important pathophysiological

mechanisms in acute (Mergenthaler et al., 2012; Quaegebeur et al., 2016) and chronic (Funfschilling et al., 2012; Mergenthaler et al., 2013) neurodegeneration and cell death mechanisms in the brain. Glucose metabolism and cell death regulation meet at mitochondria (King and Gottlieb, 2009) and glucose-metabolizing enzymes have been shown to be involved in cell death regulation under various circumstances (Buchakjian and Kornbluth, 2010; Mergenthaler et al., 2012, 2013; Andersen and Kornbluth, 2013) as well as apoptosis-regulating proteins to influence glucose metabolism (Gimenez-Cassina and Danial, 2015).

Methods for the cultivation of rodent primary neurons and other brain cells have been established for decades (Bottenstein and Sato, 1979; Brewer et al., 1993). Given the fundamental role of cellular model systems for biological discovery and investigating molecular and cellular mechanisms in brain function, and the role the extracellular environment plays in determining intracellular function (Bosche et al., 2016; Guo et al., 2016), we here investigated the role of the culture microenvironment on the preferential use of distinct metabolic pathways in neurons.

MATERIALS AND METHODS

Primary Neuronal Cultures

Wistar rats were handled in accordance with institutional guidelines and with permission of the *Landesamt für Gesundheit und Soziales (LAGeSo), Berlin*. Brains of day 17 Wistar rat embryos (E17) were isolated and cortices were dissected and seeded with a density of 175,000 cells per cm^2 and cultured for 9 days as described (Mergenthaler et al., 2012) unless otherwise stated. Briefly, primary cortical neurons were seeded in Neurobasal medium (NBM; Invitrogen, Thermo Fisher Scientific) supplemented with 25 μM glutamate, 0.5 mM L-glutamine, and a serum-free supplement (B27, N2, Invitrogen, Thermo Fisher Scientific, or GS21, MTI-GlobalStem). B27, N2, and GS21 are based on published formulations (Bottenstein and Sato, 1979; Brewer et al., 1993; Chen et al., 2008); see **Table 1** for details on their composition. The medium was partially replaced every 4 days with NBM supplemented with 0.5 mM L-glutamine and serum-free supplement. Alternatively, neurons were cultured in BrainPhys medium instead of NBM which was prepared in house based on the published formulation (Bardy et al., 2015) prior to its commercial availability (see **Table 2** for the exact formulation used herein). The bicarbonate concentration was slightly decreased to 26 mM to adjust the pH to 7.40. Primary cortical neurons were seeded in BrainPhys supplemented with 25 μM glutamate and a serum-free neuronal supplement (B27, N2, or GS21), and the medium was partially replaced during the cultivation period as described above.

Metabolic Deprivation Experiments

On day 9 of cultivation, neuronal cultures were washed twice with PBS and then incubated under anoxic conditions (0% O_2, 37°C, 5% CO_2) in a Concept-400 hypoxia workstation (Ruskinn Technologies) or under normoxic conditions (21% O_2, 37°C, 5% CO_2) for 8 h in BSS_0 (116 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO4, 1 mM NaH_2PO_4, 26.2 mM $NaHCO_3$, 10 μM glycine, 1.8 mM $CaCl_2$, 10 mM HEPES pH 7.4) as described (Mergenthaler et al., 2012). After the experimental procedures, the supernatant was analyzed directly or medium was added to the cells for 24 h until neuronal cell death was quantified by measuring lactate dehydrogenase (LDH) release.

Lactate Dehydrogenase (LDH) Release Assay

Cell death was evaluated 24 h after metabolic deprivation by measuring LDH release from primary neurons in a coupled spectrophotometric assay as previously described (Niu et al., 2017). Briefly, 50 μl supernatant or 25 μl LDH standard (500 U/l, DiaSys Greiner) for data normalization were mixed with 200 μl of 212 μM β-NADH in 33.3 mM KH_2PO_4 and 66.7 mM K_2HPO_4 (pH 7.4), and then 25 μl of 22.7 mM pyruvate in 33.3 mM KH_2PO_4 and 66.7 mM K_2HPO_4 (pH 7.4) to start the reaction. The reduction of β-NADH to NAD+ is proportional to the LDH activity and was measured by absorbance at 340 nm on an MRX revelation (Dynex Technologies) plate reader at room temperature (RT). Total LDH release was measured after incubating neurons with Triton X-100 for 30 min at 37°C (final concentration: 0.5% v/v) and a second measurement with 25 μl supernatant or 25 μl LDH standard was performed. All data were normalized to the total LDH release measurements.

Lactate Measurements

The colorimetric L-Lactic Acid Assay Kit (AAT Bioquest) was used according to the manufacturer's instructions to measure lactate levels in the media of rat brain cortical neurons cultured for 9 days. Briefly, 50 μl sample was mixed with 50 μl assay buffer and absorbance was measured after an incubation period of 90 (**Figure 2A**) or 30 min (**Figure 2B**) at 550 nm on an MRX revelation (Dynex Technologies) plate reader at RT. Measurements of each well were normalized to its total protein content as quantified with a BCA Assay Kit (Thermo Fisher Scientific).

Metabolic Flux Analysis

Metabolic flux was measured using the Seahorse XFe96 Extracellular Flux Analyzer (Seahorse Bioscience, Agilent Technologies). Appropriate cell numbers and compound concentrations were titrated in preliminary experiments (data not shown). The cell seeding density and compound concentrations given below represent conditions with the optimal dynamic range in our preliminary assays. Neurons were seeded at a density of 20,000 cells/well, and cultured for 9 days in NBM or BrainPhys as described above in the presence of the different neuronal supplements (B27, N2, or GS21). Two types of metabolic analyses were performed: the cell mito stress test to assess respiratory activity, and the glycolysis stress test to assess glycolysis (**Figure 3**). Both assays were performed in DMEM-D5030 (Sigma–Aldrich) containing 143 mM sodium chloride and 2 mM L-glutamine (pH 7.35–7.40). The medium was supplemented with 10 mM glucose for the cell mito stress test. Prior to the assays, cultures were washed with the respective

medium. To characterize the effects of B27, N2, and GS21 on metabolic flux, neurons were cultured with the respective supplements in the media indicated in the figure legends. The cell mito stress test and glycolysis stress test were performed in the absence or presence of the different supplements. For the latter, the supplements were individually added to the assay medium as indicated in the figure legends so that this incubation was performed in the presence of the same supplement as the cultivation period. Neurons were pre-incubated in this medium for 1 h at 37°C (atmospheric CO_2) before the start of the measurements. For the cell mito stress test, the sensor cartridge was loaded with 0.75 μM oligomycin (Sigma–Aldrich), 0.75 μM carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP, Sigma–Aldrich), 1 μM rotenone (Sigma–Aldrich), and 1 mM antimycin A (Sigma–Aldrich), and for the glycolysis stress test with 10 mM glucose,1 μM oligomycin, and 100 mM 2-deoxy-D-glucose (2-DG; Carl Roth). These reagents were consecutively injected into each well in this order. Measurements were taken 20 min after pre-incubation and 6, 12, and 18 min after each injection with 3 min mixing intervals in between prior to the next injection. For analysis and depiction in the figures, we used the following measurements after each injection: for the cell mito stress test basal 3, oligomycin 3, FCCP 1, and rotenone/antimycin 2; for the glycolysis stress test basal 3, glucose 3, oligomycin 3, and 2-DG 2. After each experiment, total protein concentration of each well was determined with a BCA Protein Assay Kit (Thermo Fisher Scientific) to normalize oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) measurements to the total protein content of each well.

TABLE 1 | Formulation of the B27, GS21, and N2 supplements.

Component	Concentration (μM)			
	B27	(B18)	GS21/NS21	N2
D,L-α-Tocopherol acetate	n/a	(2.1)	2.1	–
D,L-α-Tocopherol	n/a	(2.3)	2.3	–
Biotion	n/a	(0.4)	–	–
Retinol, all *trans*	–	(0.3)	0.3	–
Retinol acetate	n/a	(0.2)	0.2	–
BSA	n/a	(37)	37	–
Catalase	n/a	(0.01)	0.01	–
Human recombinant insulin	n/a	(0.6)	0.6	0.8609
Human transferrin (apo-)	n/a	(0.062)	–	–
Holo-transferrin	–	–	0.062	10
Superoxide dismutase	n/a	(0.077)	0.077	–
Corticosterone	n/a	(0.058)	0.058	–
D(+)-Galactose	n/a	(83)	83	–
Ethanolamine	n/a	(16)	16	–
Glutathione (reduced)	n/a	(3.2)	3.2	–
L-Carnitine	n/a	(12)	12	–
Linoleic acid	n/a	(3.5)	3.5	–
Linolenic acid	n/a	(3.5)	3.5	–
Lipoic acid (thioctic acid)	–	(0.2)	0.2	–
Progesterone	n/a	(0.02)	0.02	0.02
Putrescine	n/a	(183)	183	100.06
Selenite	n/a	(0.083)	0.083	0.0301
T3 (triiodo-L-thyronine)	n/a	(0.0026)	0.0026	–

Note that while the composition of B27 has been published (Brewer et al., 1993), the exact concentrations of its components are not known (indicated by n/a). The table therefore lists the concentrations of the components of B18 in parentheses (Brewer and Cotman, 1989), a precursor to B27, which were retained therein. This may provide a general idea on the comparability of B27 to the other supplements. Likewise, the manufacturer of GS21 states that its formulation is based on the published formulation of NS21 (Chen et al., 2008). The formulation of N2 is available from the manufacturer and corresponds to its original publication (Bottenstein and Sato, 1979). The concentrations shown in the table correspond to the final concentrations in the culture medium.

TABLE 2 | Adapted formulation of BrainPhys medium (Bardy et al., 2015) as used in this study.

BrainPhys component	Concentration (mM)	Company
Sodium chloride	121	Carl Roth
Potassium chloride	4.2	Merck
Calcium chloride	1.1	Merck
Magnesium sulfate	1.0	Sigma
Ferric nitrate	0.000124	Thermo Fisher Scientific
Zinc sulfate	0.0015	Thermo Fisher Scientific
Sodium bicarbonate	26	Sigma
Sodium phosphate dibasic	0.5	Carl Roth
Sodium phosphate monobasic	0.45	Sigma
Glycine	0.002	Carl Roth
L-Alanine	0.002	Carl Roth
L-Serine	0.002	Carl Roth
L-Alanyl-L-glutamine	0.5	Carl Roth
L-Arginine hydrochloride	0.3	Carl Roth
L-Asparagine-H_2O	0.05	Thermo Fisher Scientific
L-Cysteine hydrochloride-H_2O	0.1	Carl Roth
L-Histidine hydrochloride-H_2O	0.15	Carl Roth
L-Isoleucine	0.416	Carl Roth
L-Leucine	0.451	Carl Roth
L-Lysine hydrochloride	0.499	Carl Roth
L-Methionine	0.116	Carl Roth
L-Phenylalanine	0.215	Carl Roth
L-Proline	0.06	Carl Roth
L-Threonine	0.449	Carl Roth
L-Tryptophan	0.0441	Carl Roth
L-Tyrosine disodium salt dihydrate	0.214	Thermo Fisher Scientific
L-Valine	0.452	Carl Roth
D-Glucose	2.5	Sigma
Sodium pyruvate	0.5	Sigma
Choline chloride	0.0641	Thermo Fisher Scientific
D-Calcium pantothenate	0.0047	Thermo Fisher Scientific
Folic acid	0.00601	Sigma
I-Inositol	0.07	Carl Roth
Niacinamide	0.0166	Sigma
Pyridoxine hydrochloride	0.00986	Carl Roth
Thiamine hydrochloride	0.00644	Carl Roth
Cyanocobalamin	0.000502	Carl Roth
Riboflavin	0.000582	Carl Roth
Hepes	5.0	Carl Roth
Phenol red	0.0215	Sigma

Statistics

Neuronal cultures from different embryos were considered as independent observations. Statistical graphing was performed by using GraphPad Prism 5.0. One-way ANOVA and Turkey's HSD *post hoc* tests were calculated for all experiments with $n \geq 3$ in GraphPad Prism 5.0 or IBM SPSS Statistics 24.0. A $p < 0.05$ was considered statistically significant. In all figures, this is represented by $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, and $^{***}p \leq 0.001$. Figures display single observations as single data points as well as mean ± SD with a whisker plot.

RESULTS

The Neuronal Culture Supplement B27 Protects Neurons from Cell Death Under Glucose Deprivation

Neurons that were depleted of glucose (i.e., no glucose present during incubation) for 8 h were rescued from cell death when B27 was added to the deprivation buffer (BSS_0), indicating that B27 might be able to support neuronal survival for a limited time under these conditions (**Figure 1A**). This effect was not seen when neurons were incubated in NBM or BSS (BSS_{25}) containing 25 mM glucose with or without B27 for 8 h. Furthermore, decreasing glucose concentrations, thereby eliciting glucose deprivation (GD), with or without addition of B27 to BSS did not have any effect on neuronal survival under normoxic conditions (**Figure 1B**). However, as before, neurons depleted of glucose were protected from cell death when incubated in the presence of B27. On the contrary, we found that B27 decreased neuronal cell death under oxygen deprivation (OD) at low glucose concentrations, although this effect did not reach statistical significance at 0.5 mM ($p = 0.49$), 1 mM ($p = 0.197$), and higher glucose concentrations (**Figure 1C**). Furthermore, B27 protected neurons from cell death after oxygen–glucose deprivation (OGD), a model of hypoxia–ischemia, when no glucose was present in the deprivation buffer (**Figure 1C**). In summary, these data indicate that B27 is able to protect neurons from cell death triggered by glucose depletion under normoxia and hypoxia, as well as at low glucose levels under hypoxia.

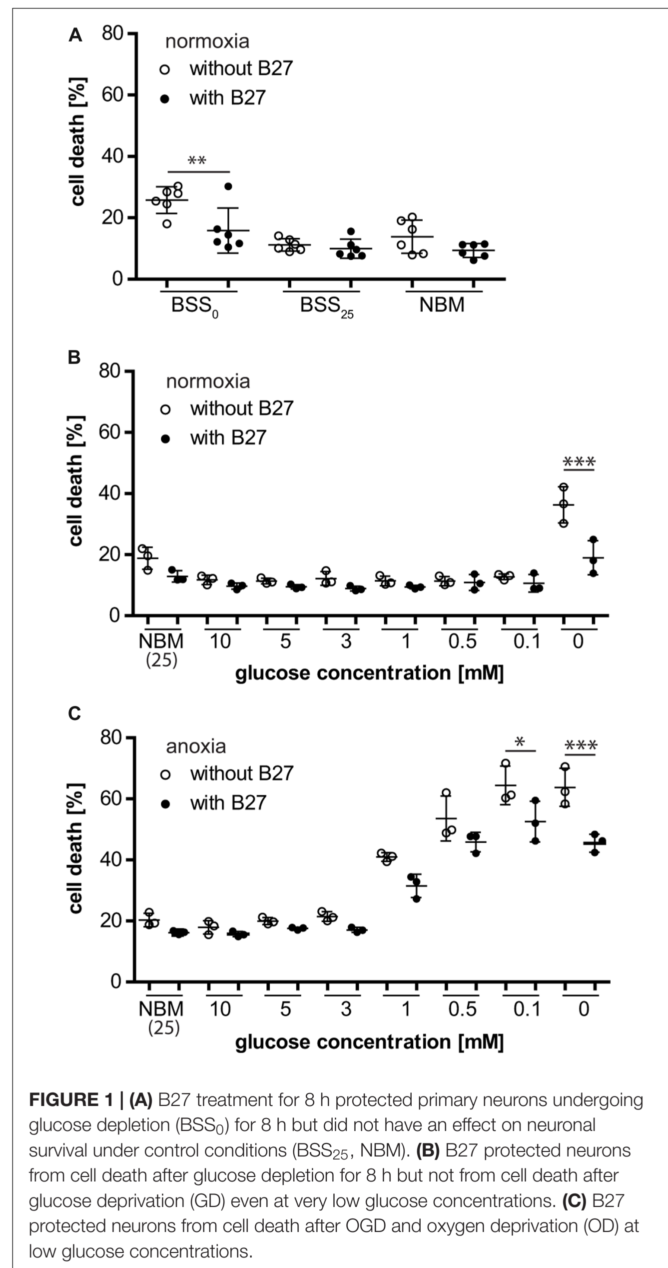

FIGURE 1 | (A) B27 treatment for 8 h protected primary neurons undergoing glucose depletion (BSS_0) for 8 h but did not have an effect on neuronal survival under control conditions (BSS_{25}, NBM). **(B)** B27 protected neurons from cell death after glucose depletion for 8 h but not from cell death after glucose deprivation (GD) even at very low glucose concentrations. **(C)** B27 protected neurons from cell death after OGD and oxygen deprivation (OD) at low glucose concentrations.

The Neuronal Culture Supplement B27 Interferes with Glycolysis

To further investigate the role of B27 in protecting neurons from cell death under metabolic deprivation, we incubated cultured neurons in the basal medium DMEM-D5030 for 98 min without glucose as described in the section "Materials and Methods" for metabolic flux analysis. As expected, addition of 10 mM glucose resulted in glucose turnover and accumulation of lactate over a period of 15 min (**Figure 2A**). However, consistent with a profound effect on neuronal glucose metabolism, addition of B27 together with 10 mM glucose completely abolished lactate accumulation in that timeframe (**Figure 2A**). Next, we added B27 to neurons undergoing hypoxia with different glucose concentrations in BSS or NBM containing 25 mM glucose (**Figure 2B**). Under all conditions, lactate accumulation was decreased by the addition of B27, although this effect did not reach statistical significance (BSS_{10} $p = 0.295$, BSS_{25} $p = 0.055$, and NBM $p = 0.881$). Together, these data suggest that B27 interferes with glycolysis.

Metabolic Flux Analysis Allows Investigating the Impact of the Culture Microenvironment on Neuronal Metabolic Function

To further investigate the metabolic changes elicited by neuronal culture conditions, we performed live cell metabolic flux analysis (**Figure 3**). This assay allows simultaneous measurements of the two fundamental pathways of glucose utilization by measuring

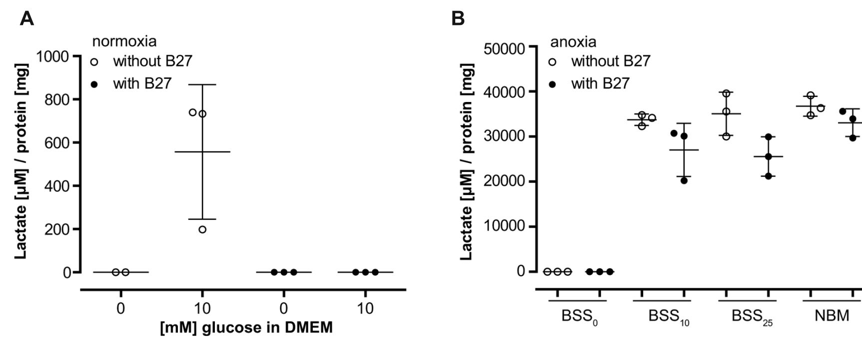

FIGURE 2 | (A) Lactate release from neurons was measured after 98 min incubation in DMEM without glucose (see section "Materials and Methods" for metabolic flux analysis) followed by a 15 min incubation with or without addition of 10 mM glucose. Co-incubation with B27 completely abolished lactate release after addition of glucose. **(B)** Lactate release from neurons was measured after 8 h incubation at anoxic conditions using BSS with or without addition of glucose and B27. Note the 30-fold difference in lactate accumulation compared to **(A)**. BSS_0, BSS_{10}, BSS_{25} – 0, 10, and 25 µM glucose in BSS. NBM – 25 µM glucose in NBM.

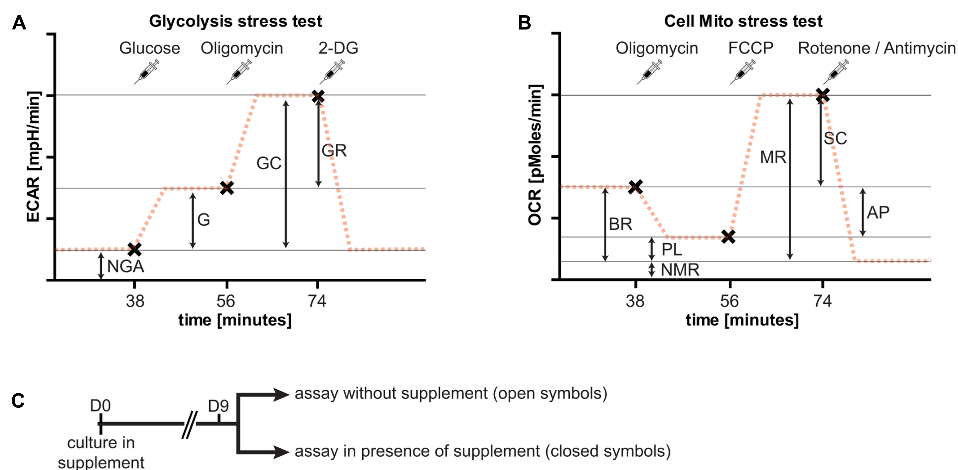

FIGURE 3 | Overview of the experimental paradigm of metabolic flux analysis. Importantly, extracellular acidification rates (ECAR) and oxygen consumption rates (OCR) are always measured at the same time. For clarity, only one of the two measurements is shown here for each assay. **(A)** The glycolysis stress test allows determination of non-glycolytic acidification (NGA), glycolysis (G), glycolytic capacity (GC), and glycolytic reserve (GR). At specified intervals (syringes, x), glucose to start glycolytic flux (38 min after start of the assay), oligomycin to block respiration (after 56 min), and 2-deoxy-D-glucose (2-DG, after 74 min) to block glycolysis are added. The addition of glucose results in lactate release-dependent increase in extracellular pH, characterized by the ECAR, and a further increase by blockade of oxidative phosphorylation. Addition of 2-DG inhibits glycolysis and decreases extracellular acidification to basal rates. **(B)** The cell mito stress test allows determination of basal respiration (BR), proton leak (PL), non-mitochondrial respiration (NMR), maximal respiration (MR), respiratory spare capacity (SC), and ATP production (AP). At specified intervals (syringes, x), oligomycin to block basal respiration by inhibiting complex V leading to decreased OCR (after 38 min), carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) to uncouple mitochondria resulting in MR (after 56 min), and rotenone and antimycin A to block complex I and III, respectively, of the respiratory chain, thereby completely inhibiting mitochondrial respiration (after 74 min) are added. **(C)** Overview of the culture paradigm used for the experiments described in **Figures 4** and **5**. Neurons are cultured in the respective supplement for 9 days. On the day of metabolic flux analysis, the medium was changed to assay medium either with or without the supplement used for culturing neurons (see section "Materials and Methods" for details).

ECAR (**Figure 3A**) as a measure of neuronal lactate production, and OCR (**Figure 3B**) as an indicator of neuronal respiratory chain activity. Addition of substrates and inhibitors in sequential order as indicated allows dynamic measurements of metabolic parameters of glycolysis and oxidative phosphorylation (see **Figure 3** and "Materials and Methods" for details). In the following, neurons were cultured in the presence of B27 or the alternative neuronal culture supplements N2 and GS21, and we performed metabolic flux measurements in the presence (closed symbols in **Figures 4**, **5**) or absence (open symbols in **Figures 4**, **5**) of the respective culture supplement (**Figure 3C**).

The Neuronal Culture Microenvironment Can Mediate Opposing Function on Neuronal Glucose Metabolism

When directly assaying glycolysis, we found that addition of B27 to the assay medium acutely inhibited glycolytic pathways by decreasing glycolysis as well as the glycolytic capacity (**Figure 4A**, circles) but did not change respiratory activity (**Figure 4B**, circles). When using the N2 supplement instead of B27 (**Figures 4A,B**, triangles), we also measured inhibition of metabolic function. Compared to the more modern

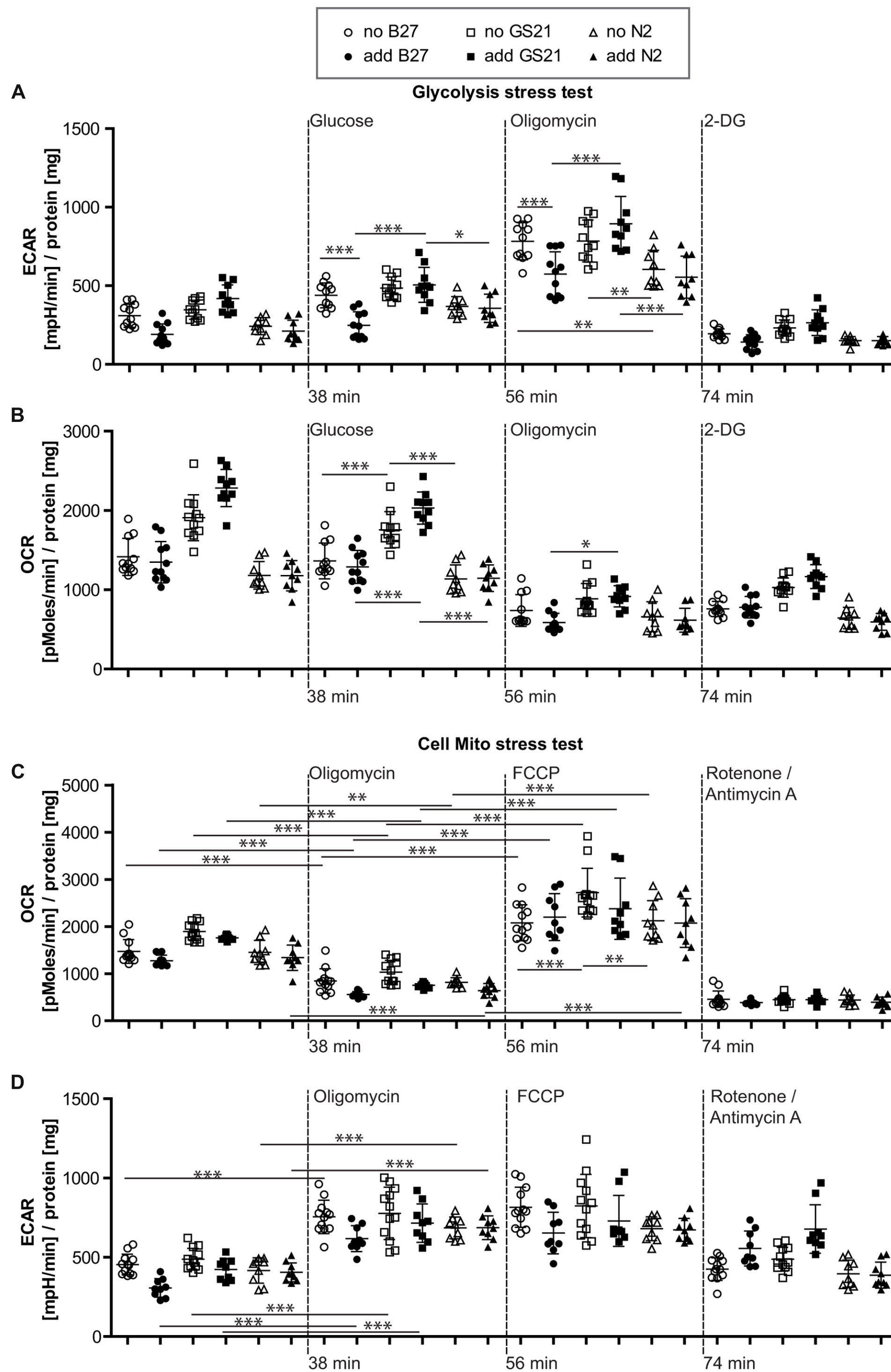

FIGURE 4 | (A,B) Glycolysis stress test after cultivation of neurons in Neurobasal medium. **(A)** Addition of B27 acutely inhibited glycolytic pathways by decreasing glycolysis as well as the glycolytic capacity (circles), while addition of N2 (triangles) did not further inhibit metabolic function. Compared to GS21 (squares), glycolytic capacity of neurons was substantially decreased with B27 or N2. **(B)** Respiratory activity was increased after cultivation with GS21 and after its addition to neurons undergoing metabolic flux analysis compared to cultivation with or addition of B27 or N2. **(C,D)** Cell mito stress test after cultivation in Neurobasal medium. **(C)** Maximal respiration was highest after cultivation with GS21 but measurements were performed in the absence of the supplement (open squares). **(D)** Glycolysis was not affected under these conditions. Open symbols indicate that neurons were cultured in the presence of the respective supplement but that was not present during measurements. Solid symbols indicate that it was also present during measurements. The time points of addition of the injection of the respective reagents after start of the metabolic flux assay are indicated with dotted lines.

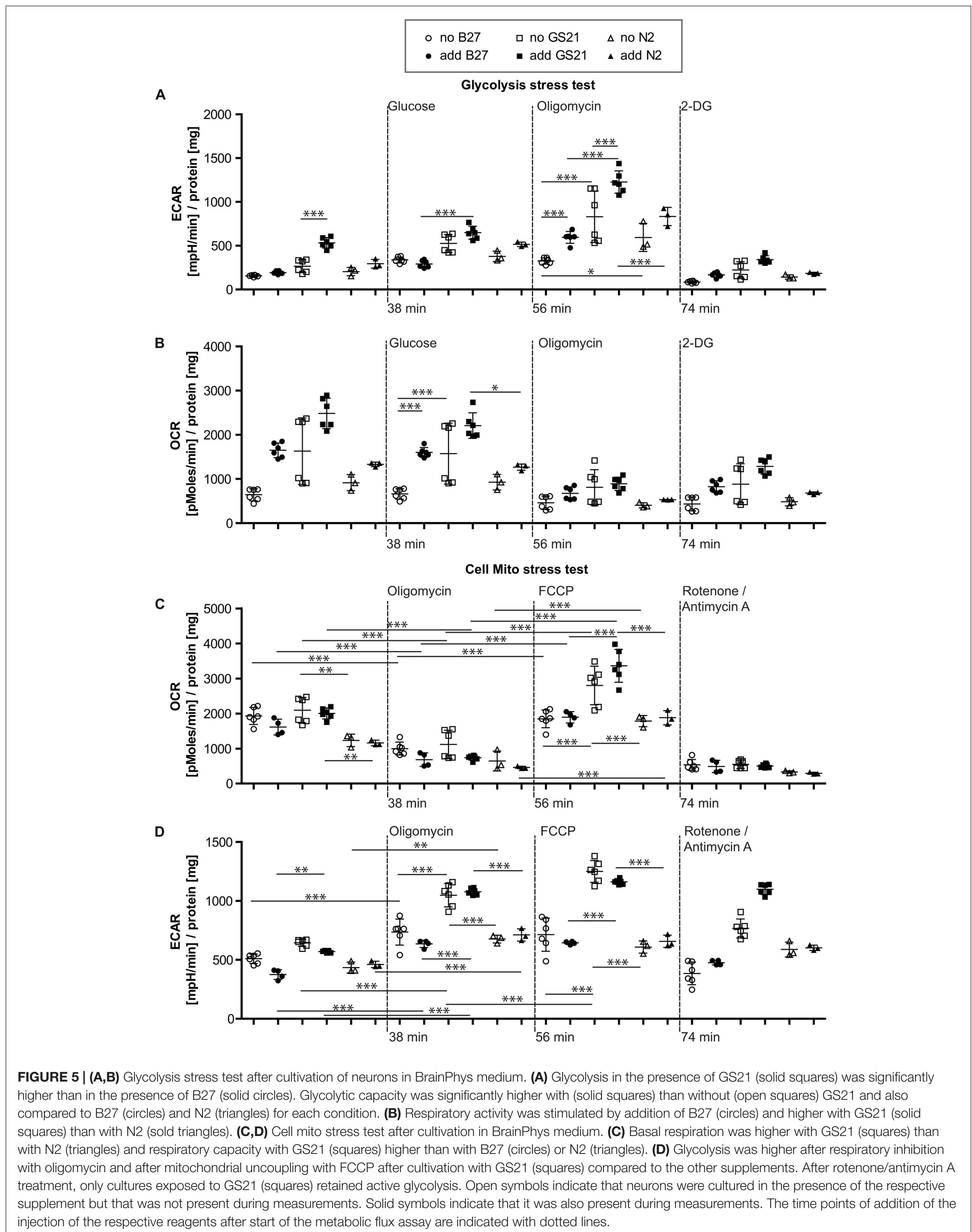

FIGURE 5 | (A,B) Glycolysis stress test after cultivation of neurons in BrainPhys medium. **(A)** Glycolysis in the presence of GS21 (solid squares) was significantly higher than in the presence of B27 (solid circles). Glycolytic capacity was significantly higher with (solid squares) than without (open squares) GS21 and also compared to B27 (circles) and N2 (triangles) for each condition. **(B)** Respiratory activity was stimulated by addition of B27 (circles) and higher with GS21 (solid squares) than with N2 (sold triangles). **(C,D)** Cell mito stress test after cultivation in BrainPhys medium. **(C)** Basal respiration was higher with GS21 (squares) than with N2 (triangles) and respiratory capacity with GS21 (squares) higher than with B27 (circles) or N2 (triangles). **(D)** Glycolysis was higher after respiratory inhibition with oligomycin and after mitochondrial uncoupling with FCCP after cultivation with GS21 (squares) compared to the other supplements. After rotenone/antimycin A treatment, only cultures exposed to GS21 (squares) retained active glycolysis. Open symbols indicate that neurons were cultured in the presence of the respective supplement but that was not present during measurements. Solid symbols indicate that it was also present during measurements. The time points of addition of the injection of the respective reagents after start of the metabolic flux assay are indicated with dotted lines.

neuronal culture supplement GS21 (squares), addition of B27 or N2 substantially decreased glycolytic capacity of neurons (**Figure 4A**). In addition, respiratory activity (OCR, **Figure 4B**) was increased after cultivation (open squares) with GS21 and after its addition (solid squares) to neurons undergoing metabolic flux analysis compared to cultivation with or addition of B27 (circles) or N2 (triangles). When we directly probed neuronal respiratory activity (**Figures 4C,D**), we found that maximal respiration was highest when neurons were cultured in the presence of GS21 but measurements were performed in the absence of the supplement (**Figure 4C**, open squares). Glycolysis was not affected under these experimental conditions (**Figure 4D**). Thus, our data indicate that the crucial supplements for culturing neurons may alter metabolic function immediately after their addition to the assay medium as well as when they are continuously present in the culture media. However, this effect is complex, as no single ingredient of B27 (**Table 1**) had a comparable effect on neuronal metabolism (Supplementary Figure 1). Importantly, culturing neurons in the presence of GS21 resulted in a denser neurite network compared to B27 or N2 (Supplementary Figure 2), thereby corroborating the results by Chen et al. (2008) that B27 can negatively affect neuronal synaptic plasticity and neurite integrity.

Finally, we investigated the metabolic function of neurons cultivated in the recently introduced BrainPhys medium (**Figure 5**), which is considered to be more physiological than previous neuronal culture media. In contrast to NBM (**Figure 4**), B27 and N2 did not inhibit glycolysis when using BrainPhys medium and the supplements were added for the measurements. However, adding GS21 during measurements still resulted in significantly higher glycolysis compared to B27 and significantly higher glycolytic capacity compared to B27 and N2 (**Figures 5A,B**). Furthermore, mitochondrial respiratory function analyzed by measuring maximal respiration was significantly higher when neurons were incubated with GS21 during OCR measurements. Finally, ATP-producing glycolysis after complete inhibition of electron transport with rotenone and antimycin A (increased ECAR, **Figure 5D**) only remained active under culture and treatment conditions using GS21.

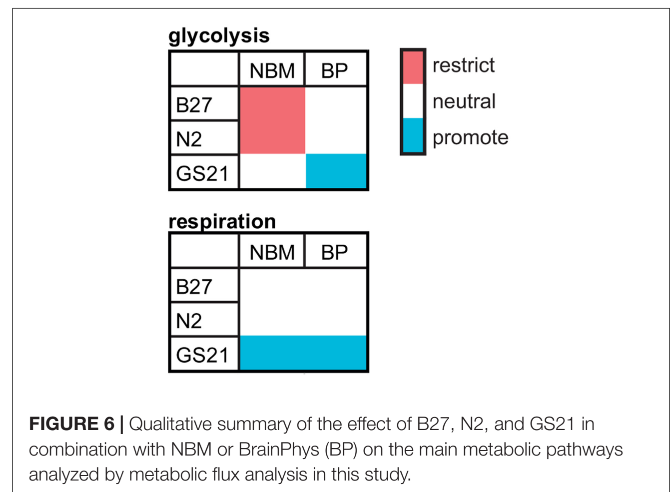

FIGURE 6 | Qualitative summary of the effect of B27, N2, and GS21 in combination with NBM or BrainPhys (BP) on the main metabolic pathways analyzed by metabolic flux analysis in this study.

DISCUSSION

Here, we describe the profound effect of the neuronal culture microenvironment on neuronal energy metabolism and neuronal survival under metabolic deprivation. We demonstrate that long-standing protocols and culture paradigms for culturing rodent primary neurons (Bottenstein and Sato, 1979; Brewer et al., 1993) have profound effects on neuronal metabolic function. To the same end, our data suggest that different culture conditions as well as acute stimulation with neuronal culture supplements may fundamentally determine the preferential use of bioenergetic pathways in neurons.

Specifically, we find that the commonly used neuronal cell culture supplement B27 can protect primary neurons from cell death after glucose depletion as well as OD under low glucose conditions and OGD. However, we find that B27 as well as the alternative neuronal supplements N2 and GS21 have different effects on neuronal energy metabolism (**Figure 6**). In that regard, B27 restricts glycolysis, an effect also seen with N2 but not with GS21. In addition, neurons cultured with GS21 had the highest maximal mitochondrial respiration, which represents the highest activity in oxidative phosphorylation neurons can achieve. Importantly, oxidative phosphorylation is the major mechanism powering neuronal activity (Hall et al., 2012). Finally, we find a complex interplay exists between neuronal culture supplements and the culture medium. When using BrainPhys medium instead of NBM, B27 and N2 did not inhibit glycolysis; however, GS21 still resulted in higher glycolytic and respiratory rates. One of the components that may be involved in mediating this effect is lipoic acid, which is one of the components of NS21 (Chen et al., 2008), the published formulation of GS21. Neither B27 nor N2 contains lipoic acid. However, the cofactor for α-ketoacid dehydrogenases, which play an important role in mitochondrial energy metabolism (Shay et al., 2009), had been present in B18 (Brewer and Cotman, 1989), the precursor to B27. Importantly, administration of lipoic acid, albeit at significantly higher concentrations than present in NS21, has been suggested to modulate glucose uptake and metabolism in the brain *in vivo* and in neurons *in vitro* through Akt/JNK signaling (Jiang et al., 2013).

In vitro systems play a crucial role for disease modeling in general and for neurobiology in particular (Mergenthaler et al., 2012; Bosche et al., 2013; Hermann et al., 2015). Therefore, defined media have been developed to support growth and maintenance of different cell types of the brain, and historically culture conditions have been optimized with regards to cellular viability, matching gene expression to *in vivo* conditions, or replicating *in vivo* phenotypes (Livesey, 2015). However, our data highlight that in addition to considering neuronal activity, metabolic parameters require equal attention in neuronal culture models.

Although controversies exist over the cell types contributing to oxidative or glycolytic glucose consumption in the brain, neuronal computation and synaptic transmission are tightly coupled to neuronal energy metabolism (Mergenthaler et al.,

2013). Furthermore, synaptic activity, which accounts for most of the brain's energy expenditure (Harris et al., 2012; Mergenthaler et al., 2013), has been suggested to genetically reprogram neuronal energy metabolism (Bas-Orth et al., 2017) further supporting the close link between neuronal energy use and computation. It is therefore not surprising that in addition to affecting neuronal metabolic function (**Figures 4**, **5**), different neuronal culture supplements and culture media also affect synapse formation, neurophysiological function, as well as neuronal viability (Chen et al., 2008; Cressey, 2009; Bardy et al., 2015). In that regard, NBM was recently shown to suppress synaptic activity (Bardy et al., 2015). Furthermore, it has been suggested that NBM may trigger excitotoxicity under certain conditions as it contains high concentrations of L-cysteine that may activate NMDA receptors (Hogins et al., 2011). Although the classical neuronal culture supplements N2 or B27 were not shown to acutely affect neuronal electrical activity when used together with BrainPhys (Bardy et al., 2015), our data suggest a significant effect on the metabolic activity of neurons elicited by these culture supplements (**Figure 5**). Furthermore, since the exact composition of B27 is kept proprietary (Cressey, 2009) despite its publication (Brewer et al., 1993), its use may be limited for certain areas of neuroscience research, such as in the field of neuroendocrinology (Roth et al., 2010).

The advent of stem cell technology to generate human neurons (Brennand et al., 2015) has boosted cell culture-based research of brain function as well as human disease modeling (Sandoe and Eggan, 2013). However, despite profound methodological advances to investigate specific metabolic pathways to (neuronal) homeostasis, physiological modeling of the neuronal microenvironment is rarely considered in current modeling concepts (Livesey, 2015). To that end, powerful experimental tools enable investigating metabolic function in brain cells on a molecular (Hung et al., 2011; Tantama et al., 2013) and cellular (San Martin et al., 2014; Lundgaard et al., 2015) level and even allow probing individual metabolites (Yaseen et al., 2013) in a functional context. Bioenergetic profiling using extracellular metabolic flux analysis (Dranka et al., 2011) provides a novel potent tool for dissecting the contribution of oxidative phosphorylation and glycolysis to neuronal function. Extracellular metabolic flux analysis measures oxygen consumption or extracellular acidification in the culture medium over time, thereby providing surrogate parameters for mitochondrial respiration or glycolytic lactate release. However, given the nature of these analytes, it is important to keep in mind that changes therein can stem from other sources than altered mitochondrial respiration or glycolysis. For example, multifactorial formation of CO_2 or conditions where pyruvate oxidation is altered can result in ECAR changes that are not a consequence of changes in the glycolytic rate (Divakaruni et al., 2014).

Imaging techniques such as positron emission tomography to investigate metabolic function in the human and rodent brain under physiological (Vaishnavi et al., 2010; Aanerud et al., 2012) and pathophysiological (Vlassenko et al., 2010; Catana et al., 2012; Heiss, 2014; Stender et al., 2015) conditions have come of age. Despite central contributions from human functional brain imaging as well as a large variety of experimental systems to understanding brain function, controversies on fundamental aspects of brain metabolism and the contribution of different cell types in the brain to metabolic function remain (DiNuzzo et al., 2010; Jolivet et al., 2010; Mangia et al., 2011; Dienel, 2012a,b; Pellerin and Magistretti, 2012; Mergenthaler et al., 2013; Lundgaard et al., 2015; Magistretti and Allaman, 2015; Machler et al., 2016), further highlighting the need for sound cellular modeling of brain function.

CONCLUSION

Together with the development of novel culture systems for rodent and human neurons (Chen et al., 2008; Bardy et al., 2015), our data provide an important foundation for future studies investigating the contribution of bioenergetic maintenance to physiological brain function or the role of deranged metabolic pathways in neurodegeneration. Investigating metabolic flux in neurons and a vast variety of other cell types has become an important tool in investigating neuronal/cellular function. Ultimately, our data support future developments in neuronal cell culture techniques and point out that careful control of the metabolic environment is an essential component in modeling brain function and the cellular and molecular pathophysiology of brain disease in culture.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Landesamt für Gesundheit und Soziales (LAGeSo), Berlin. The protocol was approved by the Landesamt für Gesundheit und Soziales (LAGeSo) Berlin.

AUTHOR CONTRIBUTIONS

JS performed experimental work, collected and analyzed the data, and compiled draft versions of this manuscript. BB discussed the data, and critically revised data analyses and drafts of this manuscript. AM allocated funding support to this project, discussed the data and analyses, and critically revised drafts of this manuscript. PM conceived and supervised all aspects of this work, analyzed and discussed the data, and wrote the paper. All authors have read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fnmol.2017.00305/full#supplementary-material

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The other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplemental Material to

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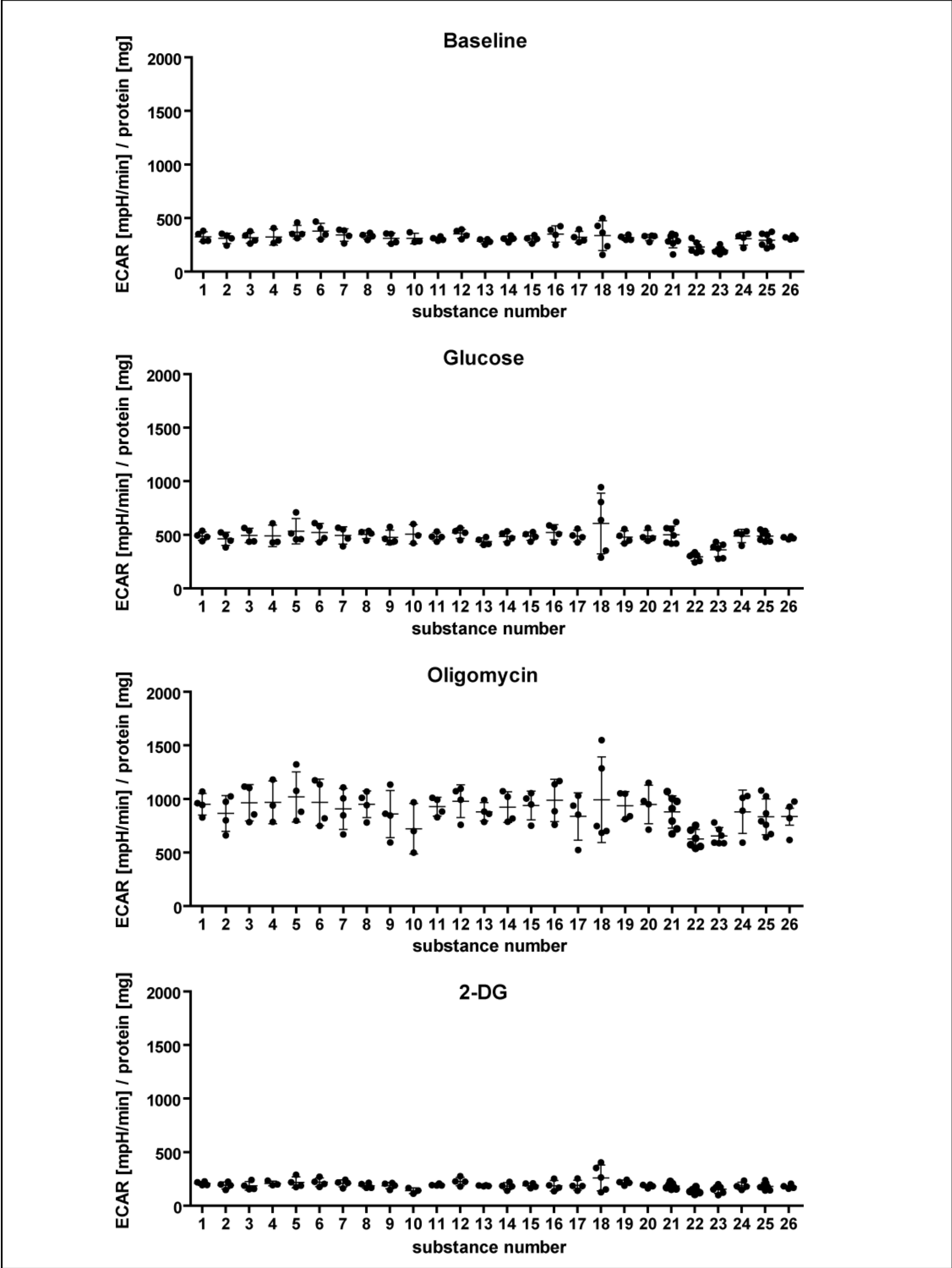
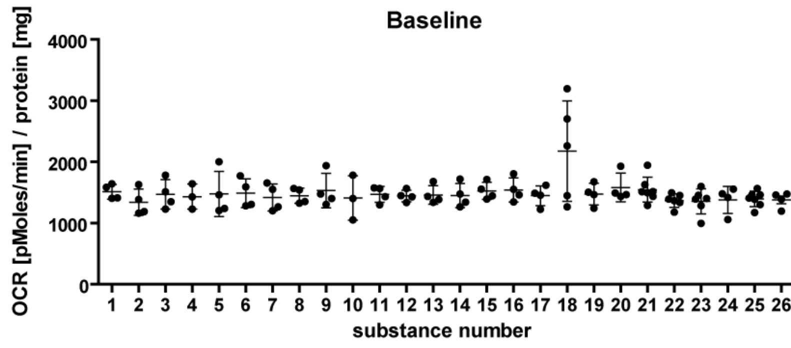
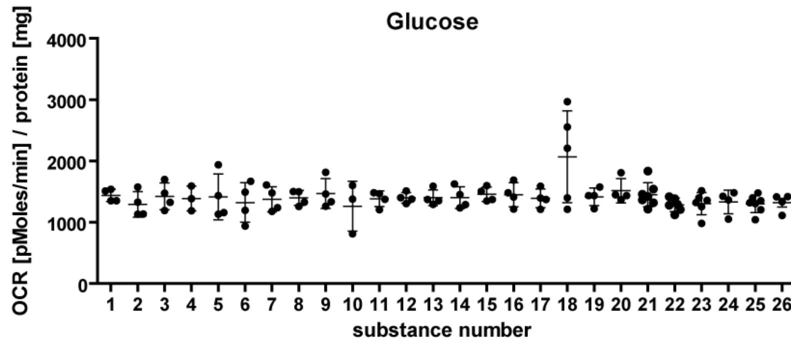
Neuronal Culture Microenvironments Determine Preferences in Bioenergetic Pathway Use.
Front. Mol. Neurosci. 10:305.; doi: 10.3389/fnmol.2017.00305

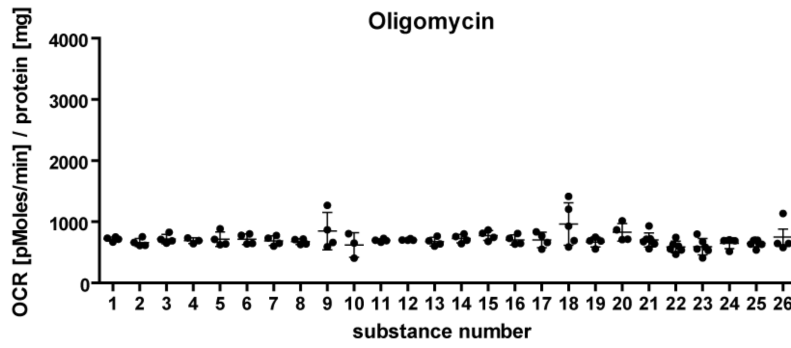
Supplemental material – Figure 1

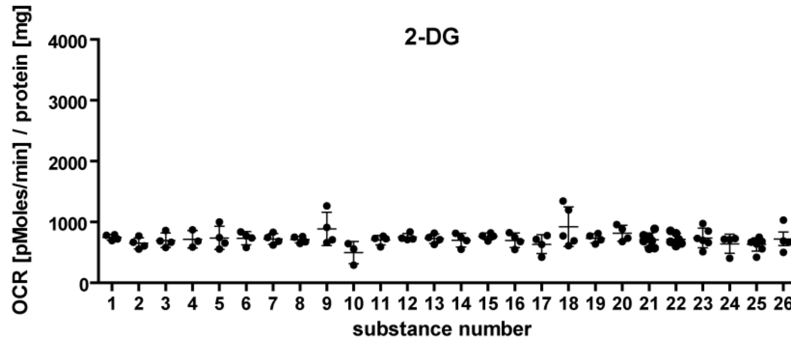
Here, the glycolysis stress test was performed after cultivation of neurons in Neurobasal media in the presence of commercial B27. For the assay, neurons were incubated with the individual component shown supplemented to the assay medium. In conclusion, individual components of B27 do not show a comparable effect on metabolic flux as the commercial B27 mix. As before, both ECAR and OCR measurements are given. See the table for each component and the concentration used. For details on the assay, see the methods section of the manuscript.




Baseline
OCR [pMoles/min] / protein [mg]
4000
3000
2000
1000
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26
substance number


Glucose
OCR [pMoles/min] / protein [mg]
4000
3000
2000
1000
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26
substance number


Oligomycin
OCR [pMoles/min] / protein [mg]
4000
3000
2000
1000
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26
substance number

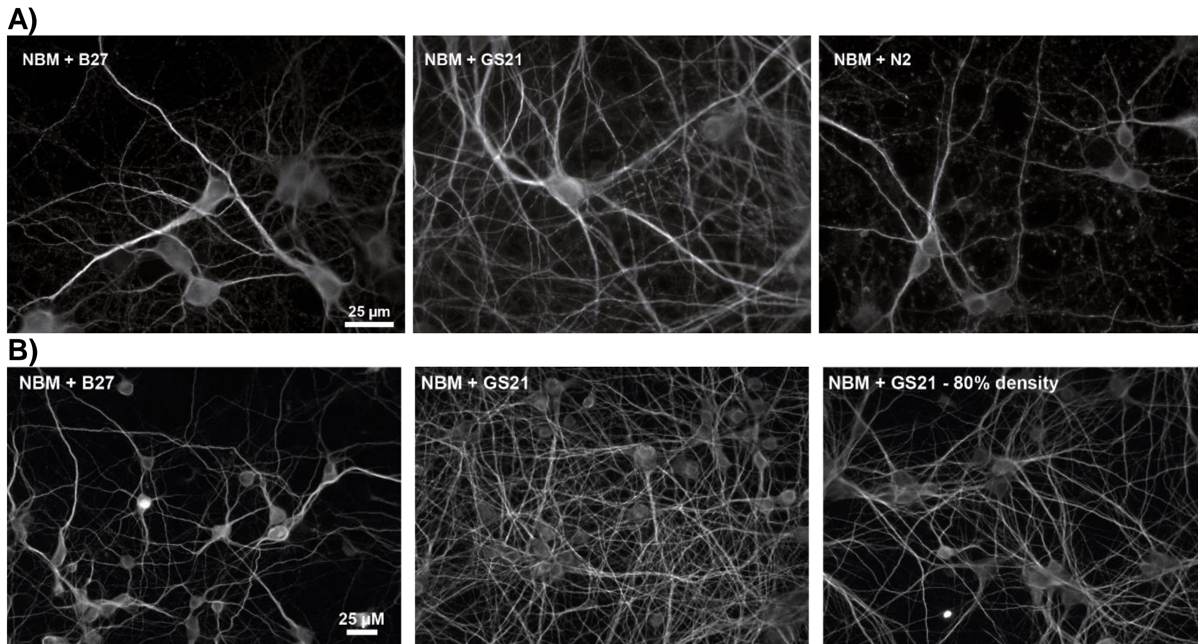

2-DG
OCR [pMoles/min] / protein [mg]
4000
3000
2000
1000
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26
substance number

Number	Component	Concentration	Solvent
1	Progesterone	0.0063 µg/ml	Ethanol
2	Linolenic Acid	1 µg/ml	Ethanol
3	Linoleic Acid	1 µg/ml	Ethanol
4	DL Alpha Tocopherol	1 µg/ml	Ethanol
5	DL Alpha Tocopherol acetate	1 µg/ml	Ethanol
6	Retinyl Acetate	0.1 µg/ml	Ethanol
7	Corticosterone	0.02 µg/ml	Ethanol
8	Human Recombinant Insulin	4 µg/ml	Ready to use
9	Human Transferrin	5 µg/ml	Aqua dest.
10	Superoxide Dismutase	2.5 µg/ml	Aqua dest.
11	Sodium Selenite	0.01435 µg/ml	Aqua dest.
12	D-Galactose	15 µg/ml	Aqua dest.
13	Ethanolamine HCl	1 µg/ml	Aqua dest.
14	Biotin	0.1 µg/ml	Aqua dest.
15	T3 (triodo-I-thyronine)	0.002 µg/ml	Sodium hydroxide
16	Putrescine 2HCl	16.1 µg/ml	Aqua dest.
17	Glutathione (reduced)	1 µg/ml	Aqua dest.
18	BSA, fatty acid free Fraction V	2500 µg/ml	Assay medium
19	Catalase	2.5 µg/ml	Aqua dest.
20	L-Carnitine HCl	2 µg/ml	Aqua dest.
21	Without B27	/	/
22	B27 (commercial)	/	/
23	B27 (self-made)	/	/
24	Ethanol (100%)	0.01 µl/ml	/
25	Aqua dest.	2.5 µl/ml	/
26	Sodium Hydroxide	0.08 µg/ml	/

Concentrations of B27 components

Substances were dissolved in aqua dest., ethanol or sodium hydroxide. Assay medium = DMEM D5030 (Sigma).

Supplemental material – Figure 2



Neurons were fixed and stained for the neuronal marker Map2 after cultivation. **(A)** Primary rat cortical neurons were cultured in Neurobasal media for 9 days in the presence of either B27, GS21 or N2 supplement. **(B)** The density of the neuronal network of GS21-cultivated neurons is higher, even when the cell number at seeding was reduced to 80% of the cell number of B27-cultured neurons.

Methods for immunofluorescence

Primary cortical neurons were washed twice with PBS, cells were fixed with 4% paraformaldehyde for ten minutes at room temperature and washed three times with PBS for five minutes and then incubated with saponine in PBS (final concentration: 0.5%) for 20 minutes at room temperature. Cortical neurons were washed again with PBS containing 0.1% saponine and incubated with a mouse-anti-MAP2 (Sigma) antibody in PBS + 0.1% saponine for one hour at room temperature. Neurons were washed three times with 0.1% saponine in PBS, and incubated with a goat-anti-mouse-Alexa Fluor546 (Thermofisher Scientific) antibody in PBS + 0.1% saponine for one hour at room temperature. Images were taken on a Leica DMI 6000 epifluorescence microscope equipped with a DFC360FX CCD camera and a HCX PL APO 63x NA1.30 objective.

Lebenslauf

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

Komplette Publikationsliste

Publikation 1

Autoren	Mergenthaler, P., Muselmann, C., **Sünwoldt, J.**, Isaev, N. K., Wieloch, T., Dirnagl, U., Meisel, A., Ruscher, K.
Titel	A functional role of the cyclin-dependent kinase inhibitor 1 ($p21^{WAF1/CIP1}$) for neuronal preconditioning
Zeitschrift	Journal of Cerebral Blood Flow & Metabolism
Veröffentlichung	2013. 33, 351 – 355. doi: 10.1038/jcbfm.2012.213
Impact Factor	5,081

Publikation 2

Autoren	**Sünwoldt, J.**, Bosche, B., Meisel, A., Mergenthaler, P.
Titel	Neuronal culture microenvironments determine preferences in bioenergetic pathway use
Zeitschrift	Frontiers in Molecular Neuroscience
Veröffentlichung	2017. 10, 305. doi: 10.3389/fnmol.2017.00305
Impact Factor	5,076

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