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ORIGINAL PAPER

Glucose, Lactate and Glutamine but not Glutamate Support Depolarization-Induced Increased Respiration in Isolated Nerve Terminals

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Abstract Synaptosomes prepared from various aged and gene modified experimental animals constitute a valuable model system to study pre-synaptic mechanisms. Synaptosomes were isolated from whole brain and the XFe96 extracellular flux analyzer (Seahorse Bioscience) was used to study mitochondrial respiration and glycolytic rate in presence of different substrates. Mitochondrial function was tested by sequentially exposure of the synaptosomes to the ATP synthase inhibitor, oligomycin, the uncoupler FCCP (carbonyl cyanide-*4*-(trifluoromethoxy) phenyl-hydrazone) and the electron transport chain inhibitors rotenone and antimycin A. The synaptosomes exhibited intense respiratory activity using glucose as substrate. The FCCP-dependent respiration was significantly higher with 10 mM glucose compared to 1 mM glucose. Synaptosomes also readily used pyruvate as substrate, which elevated basal respiration, activity-dependent respiration induced by veratridine and the respiratory response to uncoupling compared to that obtained with glucose as substrate. Also lactate was used as substrate by synaptosomes but in contrast to pyruvate, mitochondrial lactate mediated respiration was comparable to respiration using glucose as substrate. Synaptosomal respiration using glutamate and glutamine as substrates was significantly higher compared to basal respiration, whereas oligomycin-dependent and FCCP-induced respiration was lower compared to the responses obtained in the presence of glucose as substrate. We provide evidence that synaptosomes are able to use besides glucose and pyruvate also the substrates lactate, glutamate and glutamine to support their basal respiration. Veratridine was found to increase respiration supported by glucose, pyruvate, lactate and glutamine and FCCP was found to increase respiration supported by glucose, pyruvate and lactate. This was not the case when glutamate was the only energy substrate.

Keywords Synaptosomes · Seahorse XF analyzer · Neurons · Glucose · Lactate

Introduction

Changes in brain glucose metabolism are attributed to many neurodegenerative diseases such as Alzheimer's disease [1, 2] and this may raise the important question, if metabolic changes are the cause or consequence of such neurodegenerative diseases [2]. Synaptosomes are membrane surrounded structures of pre-synaptic vesicles that retain metabolic function, mitochondrial function, membrane excitability, receptors, ion channels and neurotransmitter exocytosis and transporters [3, 4]. Thus, synaptosomes offer the possibility of studying neuronal metabolism in an acute preparation and of various ages [5, 6] and genetic backgrounds of experimental animals.

Synaptosomes have been extensively used to study different metabolic pathways [5–8], neurotoxicity [9] or epilepsy [10] exemplified by the use of ^{14}C-labeled substrates to measure [14]CO_2 production [7] and incorporation of radioactivity into amino acids [6, 11]. Thus, synaptosomes are able to use glutamine as substrate for synthesis of glutamate,

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aspartate and GABA, as shown by ^{15}N-labeling experiments [11]. McKenna and colleagues have shown that synaptosomes isolated from brains from weanling and adult rats are able to utilize a variety of substrates and they have provided evidence that tricarboxylic acid (TCA) cycles with different activities exist in multiple compartments [7]. Two studies have used synaptosomes as model system to study respiration in genetically modified mice in the presence of 15 mM glucose and 10 mM pyruvate [10, 12].

Glucose availability and metabolism might change during neurodegenerative diseases and/or aging [2]. Thus, it is important to know, how neurons adapt and metabolically react to alterations in the availability of substrates to support mitochondrial respiration. Synaptosomes have been reported to readily use glucose as well as pyruvate as substrate to support mitochondrial respiration [5, 13]. Lactate accumulates and is oxidized to CO_2 in synaptosomes [14] but how lactate supports mitochondrial respiration in synaptosomes is not known.

Glutamate is mainly taken up by brain astrocytes subsequent to neurotransmission and it is known that astrocytes use a considerable amount of glutamate for oxidation [15–18]. However, the glutamate transporter Glt-1 has been shown to be important for glutamate uptake into pre-synaptic terminals [19, 20] suggesting that glutamate plays indeed an important role as energy substrate in pre-synaptic structures. Thus, also neurons take up glutamate after release [19] and may use part of it to sustain their energy needs as reported for astrocytes [15–18]. In addition, neurons use glutamine provided by astrocytes to replenish glutamate storage for subsequent neurotransmitter release [21, 22]. Cultured neurons and synaptosomes use glutamate and glutamine as oxidative substrates in the TCA cycle [22, 23], however, they were metabolized to different extents. In the present study, glutamate and glutamine were included as substrates to determine to which extent these two substrates may be able to support synaptosomal respiration.

Synaptosomes can readily be used to measure oxygen consumption and extracellular acidification/lactate release (as a measure of glycolysis) by the Seahorse Bioscience XFe24 analyzer [5, 13]. We have adapted the previously published protocol to the Seahorse Bioscience XFe96 analyzer and established a protocol to measure the different aspects of mitochondrial respiration. We have employed this protocol to investigate the ability of synaptosomes to respire on different substrates. As reported before, synaptosomes respire on glucose as well as on the mitochondrial substrate pyruvate [5]. Furthermore, synaptosomes are able to use lactate, glutamate and glutamine to support basal respiration. The ATP synthase-dependent respiration induced by veratridine was elevated to a higher extent supported by pyruvate compared to glucose or lactate as substrate.

Synaptosomes were not able to sustain ATP synthase-dependent respiration after stimulation by veratridine when glutamate or glutamine was supplied as the only substrates. In summary, the present study shows for the first time that synaptosomes are able to use lactate, glutamate and glutamine as substrates for mitochondrial respiration, but among them only lactate was able to support increased respiration by veratridine or uncoupling by FCCP and glutamine was able to support only increased respiration by veratridine, but not uncoupling by FCCP.

Materials and Methods

Materials

Sucrose, dithiothreitol (DTT), carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP), phenol red, polyethylenimine, bovine serum albumin (BSA), D-(+)-glucose, L-glutamate, L-glutamine, lactic acid, veratridine, rotenone, antimycin A and oligomycin were purchased from Sigma Aldrich (St. Louis, USA). Ethylenediaminetetraacetic acid (EDTA) and Tris base were from Bie & Berntsen A/S (Rødovre, Denmark). Percoll was obtained from GE Healthcare (Uppsala, Sweden). All other chemicals used were of the purest grade available from regular commercial sources.

Isolation of Nerve Terminal Endings

Isolation of nerve terminal endings from mouse brain (NMRI mice, older than 8 weeks, Janvier, France) was performed by a modification of a previously published method [24]. The mice were sacrificed by cervical dislocation and the brain was excised and placed in 3 mL ice-cold sucrose/ EDTA buffer (0.32 M sucrose, 1 mM EDTA, 5 mM Tris base; pH 7.4) in a 15 mL Teflon/glass homogenizer (Wheaton, Millville, USA) and homogenized by 7 up and down strokes at 700 rounds per minute. The homogenate was transferred to a 50 mL centrifugation tube and centrifuged at 800g for 10 min at 4 °C (pre-cooled, Beckman Coulter, JA-20 rotor, Krefeld, Germany). The supernatant was diluted to a final volume of 8 mL with ice-cold sucrose/ EDTA buffer and DTT was added to a final concentration of 250 µM. The diluted supernatant (2 mL) was layered on top of a Percoll gradient (2.5 mL of each 3, 10 and 23 % in ice-cold sucrose/EDTA buffer containing 250 µM DTT) and centrifuged at 31,400g for 5 min at 4 °C. The layer containing synaptosomes (above 23 % Percoll) was removed with a glass pipette, carefully re-suspended in 20 mL ice-cold sucrose/EDTA buffer and centrifuged at 20,000g for 15 min at 4 °C. The pellet was transferred to an Eppendorf tube and

carefully re-suspended in 500 µL ice-cold sucrose/EDTA buffer. The presence of synaptosomes containing pre-synaptic vesicular structures and mitochondria was confirmed by electron microscopy (data not shown).

Protein Determination

The synaptosomal protein content was determined by the Bradford protein assay (Sigma Aldrich, St. Louis, USA) according to the manufacturer's instructions. BSA was used as standard protein.

Seahorse Bioscience XFe96 Extracellular Flux Analyzer

Synaptosomes were analyzed by a modification of the previously published method [5]. Synaptosomes were diluted in salt solution (1.3 mM $CaCl_2$, 120 mM NaCl, 3.4 mM KCl, 0.4 mM KH_2PO_4, 1.2 mM $NaSO_4$, 2 mM $MgSO_4$, pH 7.4) and 8 µg/well were aliquoted in 92 polyethylenimine-coated wells [1:15,000 dilution of a 50% solution (Sigma Aldrich, St. Louis, USA) in milliq H_2O over night] of a XF 96-well plate and centrifuged at 3400g for 20 min at 4 °C for attachment of synaptosomes to the plate bottom. Plates were used immediately for experiments. The Seahorse Bioscience XFe96 analyzer (Seahorse Bioscience, Copenhagen, Denmark) was operated at 37 °C and each measurement cycle consists of 1 min mixing, 1.5 min waiting time and 3 min data acquisition. Before each injection, two measurement cycles were performed and reagents were injected in the appropriate volume of a tenfold concentrated stock solution. After the last injection per experiment, three measurement cycles were performed. Oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) express mean rates of the linear decreases of oxygen (mmHg) and pH over 3 min data acquisition time (12 individual data points). OCR and ECAR were calculated by the Wave software (Seahorse Bioscience).

The respiration parameters were calculated as follows from the mean of two individual OCR measurements:

OCR (initial respiration) = OCR (basal respiration) − OCR (rotenone/antimycin A)
OCR (ATP-dependent respiration) = OCR (basal respiration) − OCR (oligomycin)
OCR (maximal respiration) = OCR (FCCP) − OCR (rotenone/antimycin A)
OCR (spare respiratory capacity) = OCR (FCCP) − OCR (basal respiration)
OCR (veratridine-dependent ATP-dependent respiration) = OCR (veratridine) − OCR (oligomycin)

The neurotoxin veratridine, which prevents the inactivation of voltage-activated sodium channels, was used to induce an increased energy demand and synaptosomal respiration as reported previously [5].

Presentation of Data

The data shown represent means ± SEM of values that were obtained in three experiments performed on independent synaptosome preparations (biological replicates), each the average of 3–8 technical replicates. Each synaptosomal preparation was obtained from one mouse brain. The statistical analysis performed is stated in the figure legends and was performed in GraphPad Prism. P > 0.05 was considered as not significant (n.s.).

Results

Mitochondrial Respiration in Isolated Synaptosomes

So far, the respiration of isolated synaptosomes was measured in the XFe24 analyzer [5, 13]. For the present investigation, the protocol was adapted to the XFe96 analyzer. The Seahorse XFe96 analyzer is a 96-well plate reader, which has the potential for high throughput screening and is therefore preferable to the XFe24 analyzer. Isolated synaptosomes were run on the XFe96 analyzer to investigate the synaptosomal OCR and the ECAR. During 63 min, synaptosomes respired constantly with a slow decline over time to about 50% of the basal OCR (100% = 5.2 ± 1.2 nmol/min/mg protein; Fig. 1a). Addition of veratridine significantly induced an increased OCR confirming an increased energy demand (Fig. 1a).

To characterize the mitochondrial respiration of isolated synaptosomes, a mitochondrial stress test protocol as recommended by the Seahorse Bioscience Company was used. After two basal OCR measurements, veratridine or control solution (solution without veratridine) was injected, followed by oligomycin to inhibit the ATP synthase. FCCP was injected to uncouple the mitochondria and as the last injection rotenone + antimycin A were employed to inhibit all mitochondrial respiration (Fig. 1b). Addition of veratridine significantly increased the ATP-dependent respiration from 1.4 ± 0.3 nmol/min/mg protein in the absence of veratridine to 4.5 ± 0.7 nmol/min/mg protein in the presence of veratridine (**p = 0.0036; Fig. 1b). Addition of FCCP increased OCR showing uncoupling of the mitochondria and addition of rotenone + antimycin A almost completely inhibited the mitochondrial respiration to 9.9 ± 5.1% of the basal respiration (Fig. 1b).

Synaptosomal respiration was measured in the presence of 10 mM glucose as substrate. To test synaptosomal respiration at lower concentrations of glucose, the mitochondrial stress test was performed in the presence of 1, 2.5, 5 and 10 mM glucose (Fig. 1c). Indeed, synaptosomes also respired in the presence of 1 mM glucose, the lowest concentration tested. The initial respiration was significantly
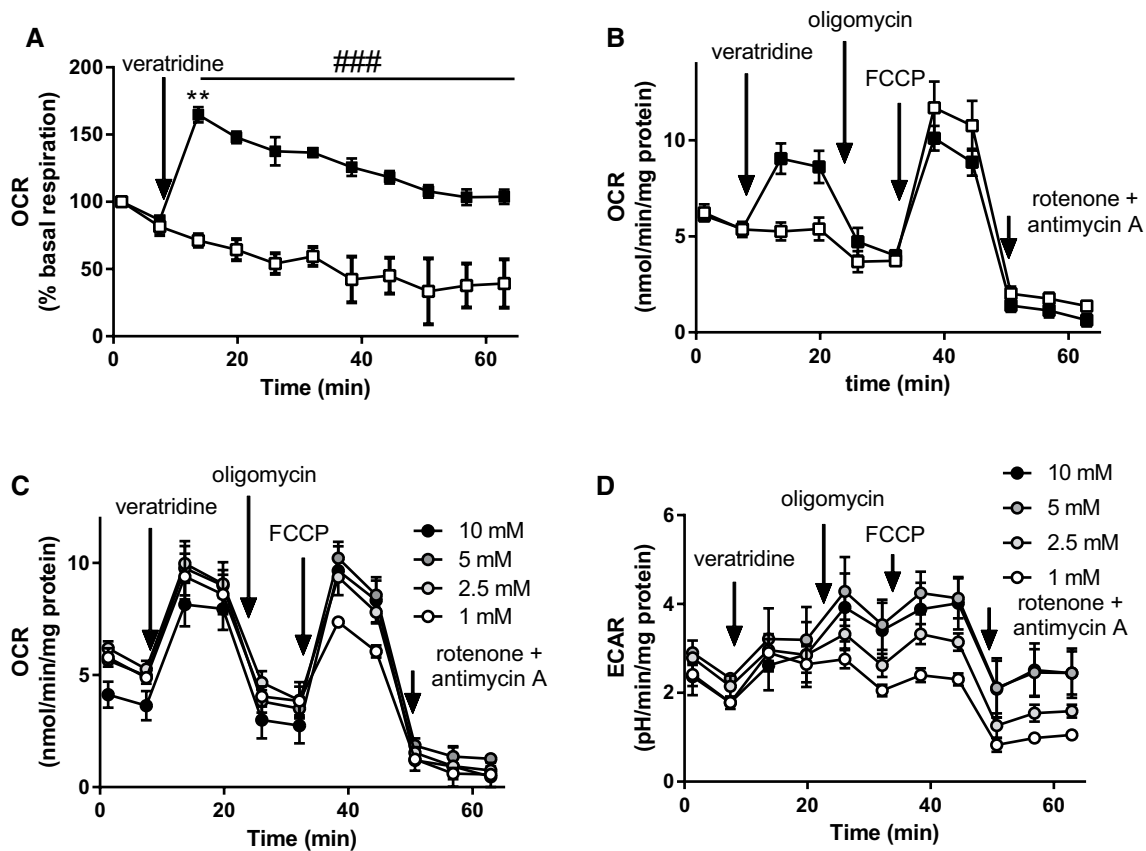
Fig. 1 Respiration of isolated synaptosomes. **a** Synaptosomal respiration (oxygen consumption rate, OCR) was determined for 63 min in the absence (*white squares*) or presence of 5 μM veratridine (*black squares*) after the first two measurement points. The basal respiration of 100% corresponds to 5.2 ± 1.2 nmol/min/mg protein (control) or 5.1 ± 1.3 nmol/min/mg protein (veratridine). Determination of synaptosomal respiration (**b, c**) and extracellular acidification rate (ECAR; **d**) during consecutive addition of 5 μM veratridine, 12 μM oligomycin, 4 μM FCCP and 2 μM rotenone + 2 μM antimycin A in the presence of 10 (**a–c**), 1, 2.5 and 5 mM glucose (**c, d**). Significant differences (ANOVA with Tukey's post-hoc test) compared to the first measurement point are indicated by **p < 0.01 and compared to the absence of veratridine is indicated by ###p < 0.001

lower in the presence of 10 mM glucose (3.0 ± 0.1 nmol/min/mg protein) compared to that obtained at 1 mM glucose (4.5 ± 0.2 nmol/min/mg protein, **p = 0.0027, Students *t* test; Fig. 1c). The respiration used for veratridine-dependent ATP production was similar at 1 mM glucose (5.1 ± 0.8 nmol/min/mg protein) and 10 mM glucose (5.2 ± 0.7 nmol/min/mg protein; Fig. 1c). The spare respiratory capacity was significantly higher at 10 mM glucose (5.1 ± 0.5 nmol/min/mg protein) compared to that obtained using 1 mM glucose (1.4 ± 0.4 nmol/min/mg protein, *p = 0.0418, Students *t* test; Fig. 1c). As the maximal respiration was highest in the presence of 10 mM glucose, this concentration was used in all further experiments.

Isolated synaptosomes contain, besides mitochondria, also all enzymes necessary for the operation of glycolysis, as shown by their ability to respire on glucose as substrate (Fig. 1). The basal ECAR of isolated synaptosomes was independent of the glucose concentration being

2.1 ± 0.1 pH/min/mg protein and 2.1 ± 0.2 pH/min/mg protein at 1 and 10 mM glucose, respectively (Fig. 1d). For all glucose concentrations tested, veratridine induced an increased ECAR whereas subsequent addition of FCCP did not further elevate ECAR (Fig. 1d). In the presence of 5 and 10 mM glucose, ECAR was significantly higher after addition of veratridine, FCCP and rotenone + antimycin A compared to the ECAR values obtained in the presence of 1 mM glucose (Fig. 1d). The increase in ECAR induced by oligomycin injection was dependent on the glucose concentration, being 0.03 ± 0.03 pH/min/mg protein (1 mM glucose), 0.13 ± 0.12 pH/min/mg protein (2.5 mM glucose), 0.71 ± 0.21 pH/min/mg protein (5 mM glucose) and 0.93 ± 0.24 pH/min/mg protein (10 mM glucose; Fig. 1c). The increase in ECAR was significantly different in the presence of 10 mM glucose compared to that obtained employing 1 mM glucose (*p = 0.0283; ANOVA).

Substrate-Dependent Respiration of Isolated Synaptosomes

Synaptosomes have been reported to be able to respire on pyruvate as mitochondrial substrate as stated in the introduction. Indeed, the initial synaptosomal respiration using 10 mM pyruvate as the substrate (6.4 ± 0.3 nmol/min/mg protein) was significantly higher than that obtained using 10 mM glucose (4.9 ± 0.4 nmol/min/mg protein) as substrate, measured at time point 7.5 min (Fig. 2a). Respiration in the presence of both pyruvate and glucose was significantly elevated compared to that using only glucose but not different from that achieved using only pyruvate (Fig. 2a). In contrast, the maximal respiration was highest in the
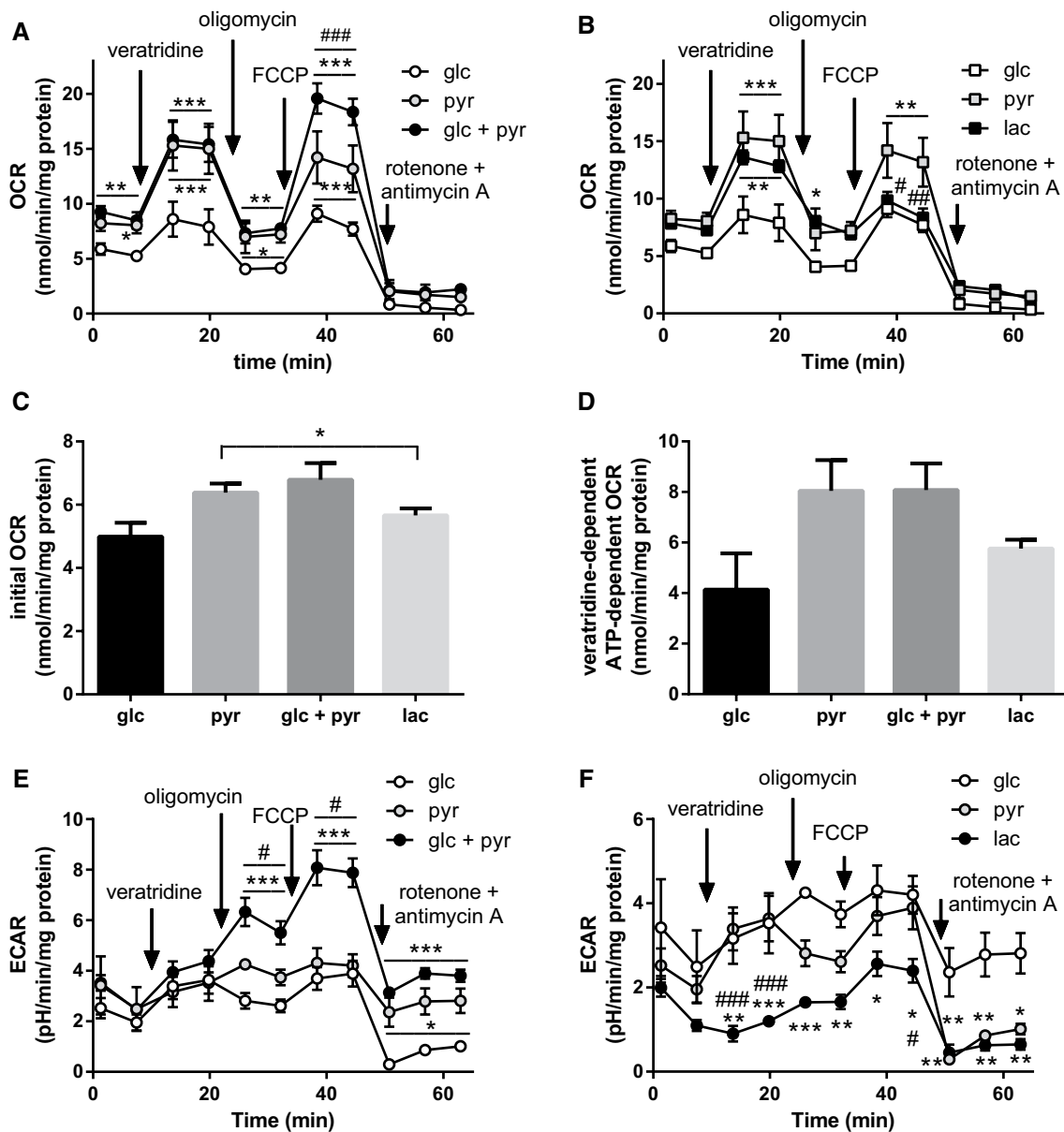

Fig. 2 Synaptosomal respiration in the presence of glucose, pyruvate or lactate. Synaptosomal respiration was determined in the presence of 10 mM glucose (**a–f**), 10 mM pyruvate (**a–f**), 10 mM glucose + 10 mM pyruvate (**a, c–e**) or 10 mM lactate (**b–d, f**). Reagents were added as shown: 5 μM veratridine, 12 μM oligomycin, 4 μM FCCP and 2 μM rotenone + 2 μM antimycin A. The graphs show the time-dependent changes of OCR (**a, b**) or ECAR (**e, f**). From data shown in (**a, b**), the average initial respiration (**c**) and the average veratridine-dependent ATP-dependent respiration (**d**) were calculated (as reported in the "Materials and Methods" section). Significant differences (ANOVA) compared to glucose as substrate are indicated by *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ (**a–c, e, f**). Statistical significances of differences were calculated (ANOVA with Tukey's post-hoc test) of glucose + pyruvate compared to pyruvate (**a, e**) or of pyruvate compared to lactate (**b, f**) and are indicated by #$p < 0.05$, ##$p < 0.01$ and ###$p < 0.001$

presence of pyruvate + glucose (16.8 ± 1.2 nmol/min/mg protein) compared to that obtained in the presence of pyruvate alone (12.0 ± 1.8 nmol/min/mg protein; Fig. 2a). Pyruvate is a direct mitochondrial substrate, which is oxidized to acetyl-CoA for further oxidation in the TCA cycle simultaneously reducing NAD^+ to NADH, the substrate for the electron transport chain. Since neurons have been reported to readily use also lactate as substrate (see "Introduction"), synaptosomal respiration was investigated in the presence of lactate (Fig. 2b). Indeed, the basal respiration as well as the veratridine-dependent respiration and the ATP-dependent respiration were comparable in the presence of pyruvate or lactate as substrates (Fig. 2b). Only the maximal respiration was significantly lower in the presence of lactate compared to that obtained in the presence of pyruvate (Fig. 2b). No difference in maximal respiration was observed in the presence of glucose or lactate as substrate (Fig. 2b).

Glutamate and glutamine can both be used as oxidative substrates by neurons (see "Introduction"), thus, we tested the ability of these substrates to support respiration in isolated synaptosomes. The synaptosomes were exposed to either 0.1 mM glutamate or 0.5 mM glutamine as substrate for respiration (Fig. 3a). Initial respiration in the presence of glutamate or glutamine was significantly higher being 5.4 ± 0.2 and 6.9 ± 0.3 nmol/min/mg protein, respectively, than the initial respiration of 4.2 ± 0.2 nmol/min/mg protein using glucose as substrate (Fig. 3a, b). In contrast, the ATP-dependent respiration in the presence of glucose, glutamate or glutamine was comparable being 2.5 ± 0.6, 2.6 ± 0.4 or 3.1 ± 0.4 nmol/min/mg protein, respectively (Fig. 3a, c). After veratridine stimulation, the veratridine-dependent ATP-dependent respiration was lower in the presence of glutamate (2.7 ± 0.2 nmol/min/mg protein) and glutamine (3.6 ± 0.1 nmol/min/mg protein) compared to glucose (4.8 ± 0.2 nmol/min/mg protein), whereas only glutamate reached the level of significance (Fig. 3e, f). The FCCP-dependent increase in respiration (Fig. 3a, e) and the maximal respiration (Fig. 3d, h) were lower in the presence of glutamate or glutamine as substrates compared to the values obtained using glucose as substrate. The effect on respiration of the presence or absence of glucose using glutamate or glutamine as substrates was tested. Indeed, the initial respiration was increased significantly in the presence of glucose + glutamine or glucose + glutamate in comparison to glucose alone, although the latter did not reach the level of significance after veratridine injection (Fig. 3b, f). The respiration used for ATP production was comparable in the presence of glucose and the presence of glucose + glutamate or glucose + glutamine (Fig. 3c, g). The FCCP-dependent respiration was strongly increased in the presence of glutamate + glucose or glutamine + glucose compared to glutamate or glutamine alone, although the level of FCCP-dependent respiration was not reached in the presence of

glutamate + glucose or glutamine + glucose compared to the level of FCCP-dependent respiration on glucose as only substrate in the absence of veratridine injection (Fig. 3a). The FCCP-dependent respiration was comparable to the values obtained in the presence of glutamate + glucose, glutamine + glucose and glucose as the only substrate after veratridine injection (Fig. 3e). Also the maximal respiration in the presence of glutamate + glucose or glutamine + glucose was significantly lower compared to that seen using glucose alone as substrate (Fig. 3d). On the contrary, after veratridine stimulation, there was only a slight decrease in maximal respiration which did not reach the level of significance (Fig. 3h).

ECAR in the Presence of Different Substrates

In the presence of pyruvate as a single substrate, the ECAR showed a similar profile compared to that seen using glucose as substrate (Fig. 2e). In the presence of glucose + pyruvate, ECAR was elevated strongly upon addition of oligomycin as well as upon subsequent addition of FCCP (Fig. 2e).

The ECAR obtained in the presence of glutamate or glutamine was as expected insignificant compared to glucose as substrate (Fig. 4a, c). The ECAR seen in the presence of only glutamate or glutamine as substrate can only originate from TCA cycle-dependent CO_2 production, not from glycolysis. Lactate is to a minor extent produced from TCA cycle derived pyruvate produced from malate, catalyzed by malic enzyme. However, the contribution via this pathway is negligible since synaptosomes produce no detectable amounts of [U-^{13}C]lactate from [U-^{13}C]glutamine [24]. The ECAR obtained in the presence of glutamate + glucose or glutamine + glucose were not significantly different from that seen in the presence of glucose alone (Fig. 4). In addition, the ECAR response upon veratridine stimulation was not affected by the presence of glutamate and glutamine in combination with glucose (Fig. 4c).

Discussion

We have investigated mitochondrial respiration and glycolytic activity of isolated mouse brain synaptosomes in the presence of different concentrations of glucose as well as pyruvate, lactate, glutamate and glutamine as substrates. We confirm that synaptosomes respire well in the presence of glucose [5, 25] and show for the first time that synaptosomes are able to respire in the presence of lactate, glutamate and glutamine as substrates. Moreover, the neurotoxin veratridine that prevents inactivation of voltage-activated sodium channels increased synaptosomal respiration as reported previously in the presence of glucose [26] or glucose + pyruvate [5, 27], showing that the synaptosomes
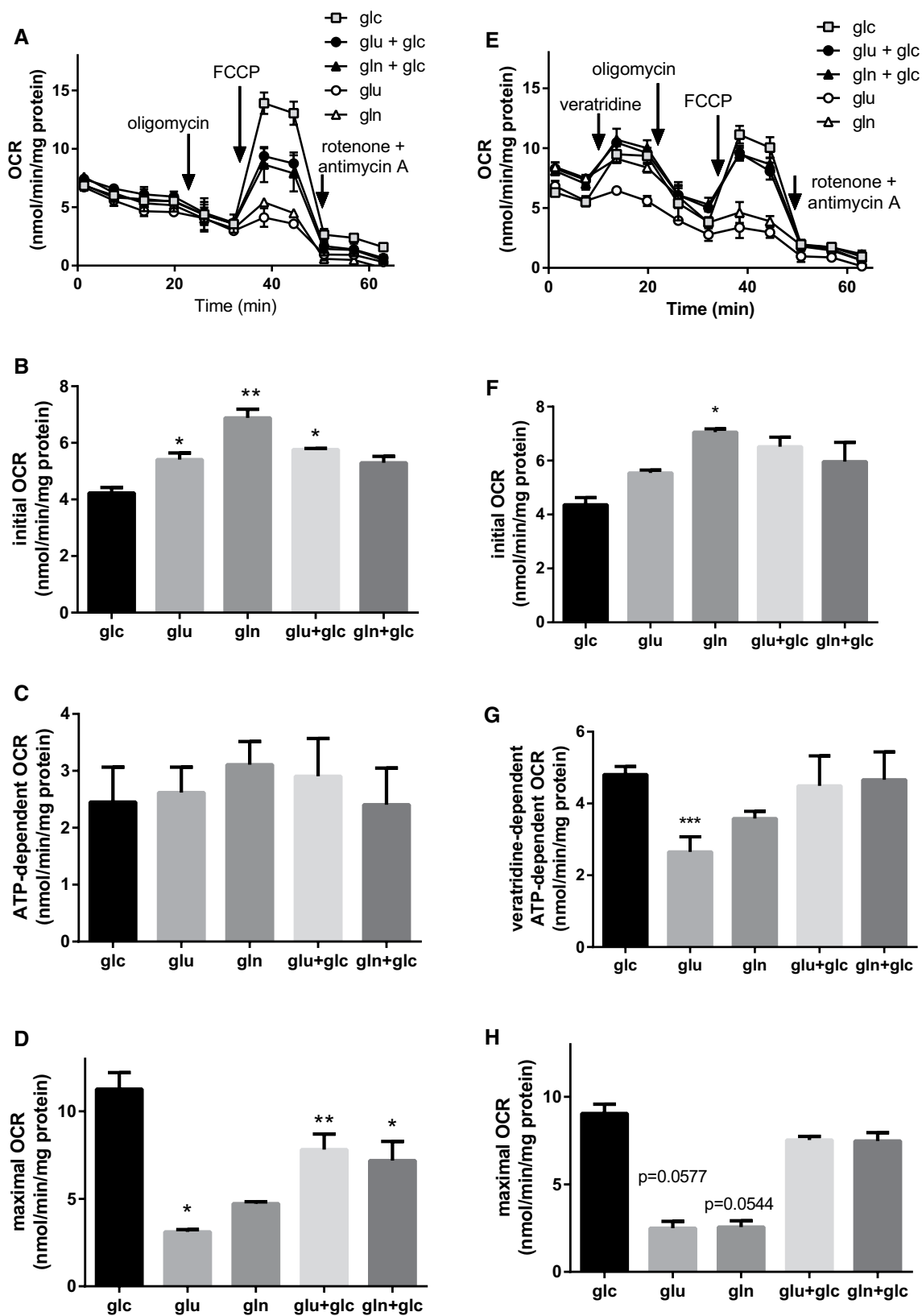
Fig. 3 Synaptosomal respiration in the presence of glucose, glutamate or glutamine as substrates. Synaptosomal OCR was determined in the presence of 10 mM glucose, 0.1 mM glutamate, 0.5 mM glutamine, 10 mM glucose + 0.1 mM glutamate or 10 mM glucose + 0.5 mM glutamine. Reagents were added as shown: 5 μM veratridine (only in **e–h**), 12 μM oligomycin, 4 μM FCCP and 2 μM rotenone + 2 μM antimycin A. The figure shows the time-dependent change in OCR (**a, e**) and the calculated initial respiration (**b, f**), ATP-dependent respiration (**c**), veratridine-dependent ATP-dependent (**g**) and the maximal respiration (**d, h**). Significant differences (ANOVA with Tukey's post-hoc test) compared to glucose as substrate are indicated by *p < 0.05, **p < 0.01 and ***p < 0.001
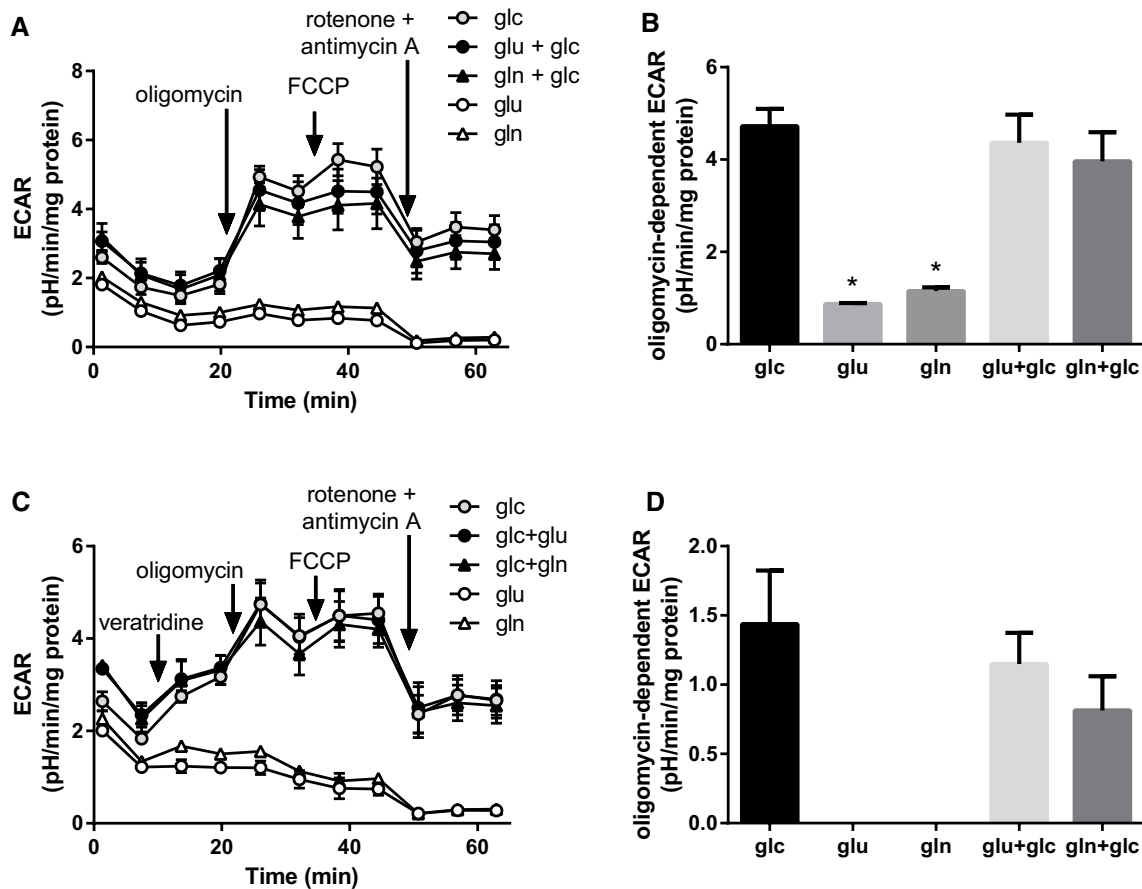
Fig. 4 Synaptosomal extracellular acidification in the presence of glucose, glutamate or glutamine as substrates. Synaptosomal ECAR was determined in the presence of 10 mM glucose, 0.1 mM glutamate, 0.5 mM glutamine, 10 mM glucose + 0.1 mM glutamate or 10 mM glucose + 0.5 mM glutamine. Reagents were added as shown: 5 µM veratridine (only in **c, d**), 12 µM oligomycin, 4 µM FCCP and 2 µM rotenone + 2 µM antimycin A. The graphs show the time-dependent change in ECAR (**a, c**) and the calculated oligomycin-dependent ECAR (**b, d**). Significant differences (ANOVA with Tukey's post-hoc test) compared to glucose as substrate are indicated by $*p < 0.05$

are able to increase their mitochondrial respiration upon stimulation.

Basal respiration of synaptosomes declined gradually to 50 % of maximum within 1 h while monitoring respiration in the Seahorse Bioscience XFe96 analyzer. This suggests a partial deterioration of the synaptosomes over time, which has been suggested before to occur at least for synaptosomal glycolysis [5]. Despite the decline in respiration over time, the synaptosomes responded well to the consecutive injections of oligomycin, FCCP and rotenone + antimycin A. Addition of oligomycin decreased the respiration, underlining the ATP-dependent part of respiration as oligomycin inhibits the ATP synthase. We observed a 50 % oligomycin-dependent decline in respiration compared to basal respiration as reported before [5]. Addition of veratridine strongly elevated the ATP-dependent part of respiration suggesting that the synaptosomes may have a low ATP demand in the absence of any stimulatory event [5, 27]. Furthermore, we show for the first time that also the substrates pyruvate, lactate and glutamine are able to support the metabolic activity

of veratridine-dependent activation. Importantly, this result stressed that synaptosomes are a structure, which can react independently to physiological stimuli. Interestingly, glutamate as substrate was not able to support veratridine-stimulated respiration, although the underlying mechanism remains to be elucidated. The veratridine-dependent increase in OCR was accompanied by an increase in ECAR [8] suggesting an increase in mitochondrial respiration as well as glycolysis to meet the increased ATP demand. Addition of the uncoupler FCCP strongly elevated the OCR independent of the absence or presence of veratridine [5]. Addition of rotenone + antimycin A almost completely inhibited the synaptosomal respiration showing that only a very small part of the observed OCR depends on non-mitochondrial oxygen consumption.

Physiological concentrations of glucose in the brain are in the low millimolar range [28], nevertheless glucose concentrations of 10–15 mM glucose are routinely used in in-vitro studies [5, 13]. In the present investigation we have tested lower glucose concentrations. Indeed, even 1 mM

glucose was able to support basal respiration. Only after FCCP-uncoupling of the mitochondria, the respiration was significantly lower in the presence of 1 mM compared to 10 mM glucose as reported before for cultured neurons [29], which may be due to a lower NADH regeneration in glycolysis subsequently available for respiration. Thus, a minimal concentration of 2.5 mM glucose is recommended to measure synaptosomal respiration. Interestingly, we observed for the first time that the veratridine-dependent increase in respiration and the oligomycin-dependent decrease were not dependent on the glucose concentration.

Pyruvate supported synaptosomal respiration and the initial respiration, the ATP-dependent respiration and the maximal respiration were higher in the presence of pyruvate than in the presence of glucose. Choi et al. [5] concluded that glycolysis may not be stable in synaptosomes at 37 °C over time, potentially due to a leakage of intermediates from synaptosomes. In addition, also the transfer of reducing equivalents from cytosol to mitochondria by the malate/aspartate shuttle [30] might limit the ability of glucose to support mitochondrial respiration compared to pyruvate as substrate [5].

In contrast to pyruvate, the synaptosomal respiration in the presence of lactate was comparable to that in the presence of glucose. Pyruvate is directly taken up by the mitochondria, whereas lactate needs to be converted to pyruvate by lactate dehydrogenase (LDH) in order to act as an energy substrate for the mitochondria. LDH concomitantly reduces NAD^+ to NADH. Thus, the utilization of lactate as an energy substrate requires continuously shuttling of redox equivalents into the mitochondria for re-oxidation of NADH to NAD^+. The malate/aspartate shuttle is the most active shuttle in the brain. However, the activity of this shuttle may limit the utilization of lactate as well as glucose compared to that of pyruvate [30].

Synaptosomes were able to respire in the presence of glutamate and glutamine. The basal respiration was increased in the presence of glutamate and glutamine. The ability of synaptosomes to use glutamate for respiration underlines the importance of glutamate as substrate for synaptic metabolism. In accordance with this finding, the glutamate transporter GLT-1 is expressed and responsible for glutamate uptake into synaptosomes [19]. In contrast, the respiration was hardly increased in the presence of glutamate and glutamine upon FCCP uncoupling. Surprisingly, even in the presence of glucose, the maximal respiration in the presence of glutamate and glutamine was significantly lowered compared to the presence of glucose alone. The reasons for this observation may be due to the different metabolic pathways of glutamate [31] and glutamine [32] uptake into synaptosomes or into the mitochondria [33]. The differences in the extracellular substrate concentrations may be one factor affecting this. Cultured cerebellar neurons consume more glutamate than glutamine, although the glutamine concentration was higher (0.5 mM) than that of glutamate (0.25 mM) in the absence of glucose [23]. In contrast, TCA cycle metabolism in isolated rat mitochondria was higher from glutamine than from glutamate as observed by metabolic mapping although the concentration of glutamate was higher than that of glutamine [34]. Mechanistically, this decreased respiration is likely to be associated with the mitochondria, as the glycolytic activity was only minimally affected by the presence of glutamate or glutamine plus glucose. Competition experiments in synaptosomes have shown that glutamine addition reduced the rate of CO_2 production from [U-^{14}C]glucose, thus, suggesting preferred use of glutamine for TCA cycle metabolism [7]. In contrast, glucose addition did not lower the CO_2 production from [U-^{14}C]glutamine [7]. These observations suggest that at least glutamine might be preferentially used in the TCA cycle over glucose and that this glutamine oxidation is to a certain extent insufficient to support maximal respiration. The specific mechanism that is responsible for this decreased respiration caused by the presence of either glutamate or glutamine in synaptosomes needs to be further elucidated. The observed differences in synaptosomal respiration in the presence of different substrates underlines, that this structure is able to use a variety of substrates. It shows at the same time, that not all physiologically possible substrate combinations support respiration equally well.

ECAR originates from the acidification by lactate plus proton release from glycolysis, but also from acidification by TCA cycle-dependent CO_2 production (in synaptosomes around 10 %) [35]. Thus, the majority of the ECAR response obtained in synaptosomes using glucose as fuel is glycolysis-dependent. The measurement of lactate release and its oligomycin-dependent increase suggest a pre-synaptic localization of monocarboxylate transporters, as pre-synaptic vesicular structures were observed by electron microscopy (data not shown). The ECAR obtained in the presence of glutamate or glutamine shows that ECAR indeed can be caused by TCA cycle metabolism and respiratory acidification. The increase in ECAR upon oligomycin addition to inhibit the ATP synthase-dependent ATP production shows that isolated synaptosomes are able to increase their aerobic glycolysis and release lactate to compensate for the decrease in mitochondrial ATP production as reported before [5]. Uncoupling of synaptosomal mitochondria by addition of FCCP further enhanced the acidification rate in the presence of glucose, but also in the presence of pyruvate as substrate suggesting that besides glycolytic lactate production, acidification is at least partially due to TCA cycle dependent CO_2 production [5, 35] or increased conversion of pyruvate directly to lactate.

In summary, the present study shows for the first time that besides glucose and pyruvate synaptosomes are able to

use lactate, glutamate and glutamine to support their basal respiration. Glucose, pyruvate and lactate as substrates support increased respiration in the presence of veratridine or the uncoupler FCCP. In contrast, glutamate and glutamine alone seem to be less efficient substrates and do not support maximal respiration while glutamine was able to support veratridine-stimulated respiration. Thus, the synaptosomal metabolism might be highly dependent on glucose or lactate availability and diminished glucose metabolism in neurodegenerative diseases might indeed affect synaptosomal metabolism.

Compliance with Ethical Standards

Conflict of Interest The authors have no conflict of interest to declare.

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