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NMDA-induced stimulation of glycolysis in developing hippocampal cell cultures

Research Article

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Abstract: Developmental changes in energy metabolism of primary hippocampal cell cultures from newborn rats were investigated during the first 3 weeks. These changes were measured by intensity of and number of cells exhibiting NAD(P)H fluorescence in response to NMDA-induced activation of neuronal activity. We observed gradual changes of stimulation-evoked NAD(P)H signaling over the first 3 weeks, such that at day 7 and 16, this stimulation is minimal, while at 5 and 12 days, it is maximal. These results describe a biphasic pattern that was similar to earlier findings from experiments investigating developmental changes in population spike amplitudes or glutamate release in young rats. Inhibition of mitochondrial respiration by KCN revealed that the NMDA-evoked stimulation of energy metabolism is mainly due to increased glycolytic activity.

Keywords: *NAD(P)H fluorescence • Cyanide • Neurometabolic coupling • Biphasic response*

1. Introduction

The brain is an organ that requires a large amount of energy for proper functioning, typically 2% to 10% of resting metabolism in animals [1]. Based on both experimental findings and theoretical considerations, it has been suggested that neuronal communication is constrained by energy supply to the brain [2]. This limitation implies that improving neuronal communication is accomplished through an increase in efficiency of neuronal network wiring instead of increasing the number of neurons. Such fine-tuning of neuronal networks is established during early postnatal brain development, and, depending on the brain system, this process may involve sensory, motor, cognitive and emotional learning events which induce the reorganization of the network architecture [3]. Since neuronal activity leads to an increased demand for metabolic energy, it can be concluded that energy metabolism also plays an important role for the development of neuronal networks.

Neurometabolic coupling describes the interplay between neurons and glia cells, that is, the directed flow of energy-rich substrates from the glia cells to the neurons. This concept, introduced by Pellerin and Magistretti [4,5], suggests that most of the glycolytic activity occurs in glia cells, whereas the neurons are more active in respiration. The transfer of energy between the two cell types is arranged by lactate. Activation of neurons stimulates glycolysis, with concomitant increased extrusion of lactate, in the glia cells.

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This hypothesis is supported by the discovery that both electrical stimulation and chemical activation *via* glutamate or kainate of neurons induce biphasic changes of NAD(P)H fluorescence, such that an initial transient decrease is followed by a longer lasting increase [6-8]. Kassischke *et al.* [8] demonstrated that this biphasic NAD(P)H response is due to two distinct monophasic metabolic responses superimposed. The initial transient decrease of NAD(P)H fluorescence originates from the neuronal dendrites (referred to as "dip"), and is caused by an elevation in oxidative phosphorylation (respiration), during which NAD(P)H is consumed, and the subsequent activation of the TCA cycle which replenishes NAD(P)H. The second response, a longer lasting transient increase of NAD(P)H fluorescence, occurs in the cytoplasm of astrocytic processes, and corresponds with an activation of glycolysis, which in turn generates large amounts of cytoplasmic NAD(P)H. This cytoplasmic NAD(P)H is then converted to $NAD(P)^+$ as lactate is produced.

Although some information about brain energy metabolism exists as indicated above, not much is known about energy metabolism in the developing brain. In order to test the hypothesis that establishment and optimization of neuronal networks is limited by the energy metabolism, we have investigated energy metabolism of primary hippocampal cell cultures during the development of synaptic networks during the first 3 weeks *in vitro*. Energy metabolism was determined as a function of fluorescence of NAD(P)H, a common indicator of glycolytic and mitochondrial activity [9-11].

2. Experimental Procedures

2.1 Cell cultures

Primary cultures of hippocampal cells were prepared from newborn rats (white Wistar) as described in Braun *et al.* [12]. These cultures were composed of equal fractions of neurons and glia cells (50%:50%). The cells were placed on a polylysine coated cover glass (12 mm diameter) and cultured during the first day in culture medium (M1) containing: 90% DMEM (Sigma), 0.45% glucose (Sigma), 40 mM $NaHCO_3$ (Sigma), 10% fetal bovine serum (Biochrom), 20 µg/ml gentamicin (Invitrogen) and 1 mM glutamax (Invitrogen). Subsequent cultivation was with culture medium (M2) consisting of 98% Neurobasal, 2% B27, 10 µg/ml gentamicin, and 1 mM glutamax (all from Invitrogen). The medium (M2) was pH stated to 7.35, and renewed every 4 days. The cells were incubated at 37°C under constant aeration with 95% O_2/5% CO_2.

We measured changes in energy metabolism in response to external perturbations over time (3-22 days *in vitro*). On the day of the experiment, the cells were taken from the incubator, and placed for 5 minutes into conditioning medium (CM) of the following composition: 129 mM NaCl (ROTH), 4 mM KCl (J.T.Baker), 1 mM $CaCl_2$ (Merck), 1 µM glycine (Merck), 10 mM HEPES (Serva), 4.2 mM D-Glucose (Sigma), pH 7.35. Osmolarity was adjusted to 315 mOsm with Sucrose (ROTH). Thereafter, the cells were transferred into a recording medium (RM), with similar composition, excepting the replacement of glycine by 0.5 mM $MgCl_2$ (Merck).

2.2 Chemical stimulation

NMD(L)A and KCN were prepared by adding to distilled water, with a new solution made for each preparation. Control experiments confirmed that injection of distilled water into the cell culture did not induce changes in cellular NAD(P)H. All concentrations of the injected stock solutions are given in the results.

2.3 Activation of neuronal activity by NMDA

Energy metabolism following chemical stimulation of neuronal activity was measured on different days of cultivation (3-22 days *in vitro*). Neuronal activation was induced by applying N-Methyl-DL-aspartic acid, NMD(L)A (Sigma), using a nanoinjector (Type nanoject from H. Saur, Germany). The tip (diameter between 5-7 µm) was placed in close proximity to the cells from which fluorescence measurements were taken. In a number of experiments, 50 µM of the NMDA receptor antagonist D(-)-2-Amino-5-phosphonopentanoic acid (D-AP5, Calbiochem) or 2 mM KCN (Merck) were added to the recording medium.

2.4 Inhibition of respiration by KCN

In order to distinguish between aerobic and anaerobic energy metabolism (*i.e.* between glycolysis and respiration), KCN was added to the solution bathing the cells. To test the effect of KCN on energy metabolism, 60 nl of 0.5 M solution was injected locally into the extracellular fluid during recording of the NAD(P)H-fluorescence. The final overall concentration of this KCN bolus was 0.1 mM. For other experiments, 2 mM KCN was added to the recording medium (see above).

2.5 Fluorescence measurement

The cell cultures were transferred into a reaction chamber mounted on the stage of an inverted fluorescence microscope (Axiovert 200 Zeiss, Jena) equipped with a 40x 1.3 NA oil-immersion objective lens (Plan-Neofluar, Zeiss).

Fluorescence images within an area of 350x400 µm were recorded *via* an image intensified CCD-camera (Proxitronic, Bensheim, Germany) with a rate of 12.5 images *per* second. Cellular NAD(P)H fluorescence was excited with a mercury lamp (HBO 103 W, Zeiss) through 365/12 nm band pass filters. The fluorescence at 460 nm was recorded through a 397 nm long pass filter. The fluorescence images yielded only diffuse shapes of cells; moreover, not all cells exhibited fluorescence. In order to locate all cells within the measuring area, additional transmission images of the cells were taken at the onset of the experiment and then overlaid on the fluorescence images (see below).

2.6 Image processing

NAD(P)H fluorescence images were imported into to a computer (PC) using a monochrome PCI frame grabber (DT3162, Data Translation). The acquisition program has been written in the graphic language LabView (National Instruments). The images were stored as TIFF format for further analysis.

Temporal dynamics of NAD(P)H fluorescence from each individual cell was investigated by gray level analysis. For this, the fluorescence images were overlaid with transmission-light images, using the GNU Image Manipulation Program (GIMP) – this enabled freehand selection of several cells within one image for gray level analysis. Consequently, we could localize all cells within the observation area and attribute the fluorescence changes to the identified cells. The gray levels of each cell exhibiting fluorescence were averaged and this was recorded as fluorescence intensity (F). This fluorescence value was normalized and then plotted as a function of time. The normalized intensity of NAD(P)H fluorescence was defined as $F' = (F-F_0)/F_0$, where F_0 is the fluorescence intensity before stimulation of the cells. Subsequent image analysis was done by several programs written in the interactive data language (IDL, RSI).

Cellular response to the chemical application was quantified by the overshoot amplitude of NAD(P)H-fluorescence (see Figure 1), and expressed as the percentage of responding cells with respect to the number of all cells in the area of observation as determined by transmission image.

2.7 Statistics

All results are presented as mean values ± S.E.M. Significant differences in the overshoot amplitude at different ages of the cultures were tested for using a Tukey One-Way ANOVA analysis.

3. Results

3.1 NMDA-induced activation of the energy metabolism

Analysis of fluorescence images clearly shows that NAD(P)H fluorescence is located only within cells, but that its intensity varies from cell to cell (Figure 1a). Upon application of NMDA, a transient increase in NAD(P)H fluorescence (about 15 seconds) was observed (Figure 1b). This response to NMDA, however, was effectively inhibited by addition of NMDA receptor antagonist D-AP5 (Figure 1c). Repeated injections of NMDA induced a similar response in NAD(P)H-fluorescence (Figure 2a). NMDA induced signals were not the results of methodological artifact (Figure 2b).

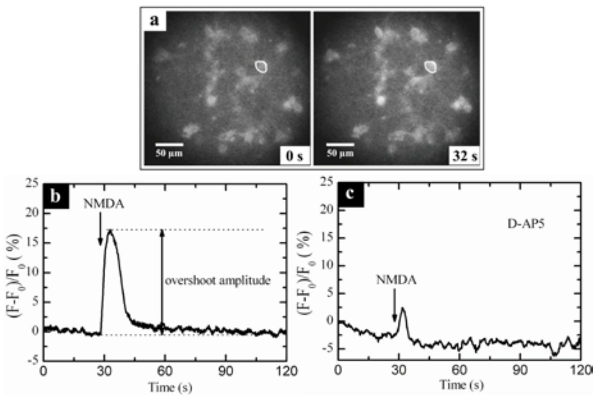

Figure 1. NMDA induced increase of cellular NAD(P)H fluorescence. (a) Fluorescence image of the cell culture at 19 day *in vitro*. The cell analyzed for temporal fluorescence changes is marked. The images shown were taken before (0 s) and after (32 s) NMDA injection. (b) Temporal changes of the normalized NAD(P)H fluorescence after injection of 60 nl of 25 mM NMDA. The time of NMDA injection is indicated by a single headed arrow. The overshoot amplitude used for all further analyses is shown. (c) Same as (b) but in the presence of 50 µM D-AP5.

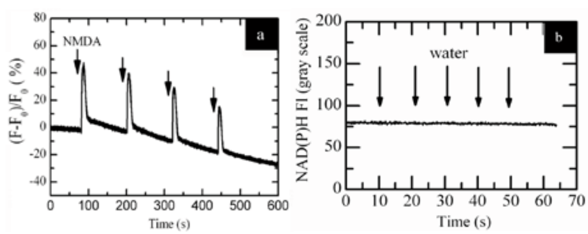

Figure 2. Repeated stimulation and control experiments. (a) The response of rat hippocampal cells to NMDA (50 mM) injection (arrows) (see text for details). (b) The response of rat hippocampal cells to the solvent of NMDA (water) as a reference or methodological control.

3.2 Optimization of NMDA stimulation

Increasing injected volume of a 50 mM NMDA solution from 9 to 74 nl induced increases in both the overshoot amplitude of NAD(P)H fluorescence and the percentage of responding cells until saturation occurs (about 40 nl, Figure 3a). Therefore, for all subsequent experiments, we chose 60 nl for a single pulse. The percentage of responding cells increased in a dose dependent fashion as concentration of NMDA increased from 12.5 mM to 25 mM; further increases (up to 50 mM) had little effect, and so to minimize toxic effects of NMDA, 25 mM NMDA was used for all following experiments. This concentration of NMDA is diluted a further 5,000 times when added to the recording buffer, and so the final averaged concentration for a single pulse was approximately 5 μM.

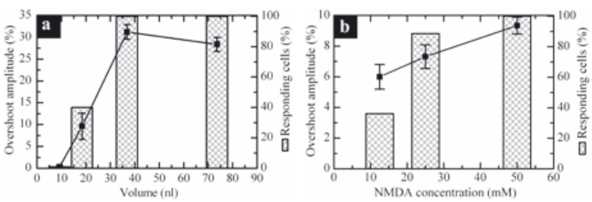

Figure 3. Optimization of the conditions for NMDA injection. (a) Effect of volume of NMDA solution: dependence of the overshoot amplitude of NAD(P)H fluorescence (line) and the number of responding cells (column) on the volume of injected NMDA solution. The concentration in the capillary was kept constant to 50 mM. The age of the cells was 7 days *in vitro*. The bars represent the mean ± S.E.M of 8-20 cells. The differences in the overshot amplitude between 9 nl and 18 nl as well as 18 nl and 36 nl were significant (P<0.005). (b) Effect of concentration of NMDA solution: dependence of the overshoot amplitude of NAD(P)H fluorescence (line) and the number of responding cells (column) at varying NMDA concentrations. The injected volume was set to 60 nl. The age of the cells was 7 days *in vitro*. The differences in the overshot amplitude between 12.5 mM and 25 mM was not significant (P>0.3), whereas it was significant between 25 mM and 50 mM (P<0.04).

3.3 Developmental changes in NMDA-induced NAD(P)H responses

The overshoot amplitude of NAD(P)H flourescence changed with the age of the cells (Figure 4a). Within 5-22 day *in vitro* (DIV), the overshoot amplitude changed in a biphasic manner with two minima at 7 DIV (7±3%) and 16 (5±2%) DIV. Mean overshoot amplitude at these minima (at 7 DIV and 16 DIV) is statistically different (P< 0.0001) from those of the maxima (5 DIV, 12 DIV and 22 DIV).

The percentage of responding cells was 74±17% at 5 DIV and 77% at 22 DIV, the times of overshoot maxima, indicating no significant increase in the number of responding cells over this culturing period (Figure 4b). After 8 and 14 DIV, only 42±14% and 21±12% cells respectively were NMDA responsive (Figure 4b). Unlike overshot amplitude, only the second minimum is significantly different from the maxima at 11 DIV and 21 DIV (P<0.03).

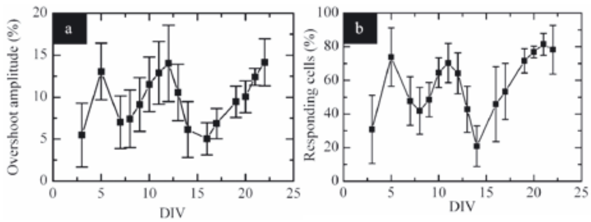

Figure 4. The effect of age of cells on metabolic activation. Cell cultures were taken at different days *in vitro* (DIV) and analyzed for NMDA-evoked stimulation of NADPH-fluorescence as shown in Figure 1b. (a) Change in overshoot amplitude as a function of age (DIV). (b) Percentage of cells responding to NMDA-stimulation plotted against time of growth. These growth dependent response of the energy metabolism could be reproduced several times, albeit with a slight temporal shift of the minima between different cultures. In order to compare the different data sets, these shifts have been corrected by adjusting the first minimum to the same time instant for all measurements (5 DIV). Error bars represent the mean ± S.E.M of up to 5 independent measurements.

3.4 Testing for metabolic performance

Age dependent changes in the cellular energy metabolism induced by NMDA stimulation could be hypothesized to occur by a number of different mechanisms. They might either reflect developmental changes in, for example, the complexity of synaptic wiring or changes in the expression of NMDA receptors or other receptor types. Alternately, they could occur due to developmental changes in energy metabolism itself during neuronal network formation. To resolve these possibilities, we applied potassium cyanide to the cells. As KCN blocks mitochondrial respiration with concomitant accumulation of NAD(P)H [8], and if there is in fact a direct relationship between energy flow (*i.e.* glucose oxidation) and mitochodrial respiration, then it follows that the increase in NAD(P)H after application of KCN would indicate the extent of this energy flow.

Similar to the response following NMDA stimulation, NAD(P)H fluorescence increased immediately after cyanide application (Figure 5a). However, in contrast to NMDA stimulation, KCN induced responses in 100% of the cells in all experiments and at all developmental stages. Moreover, the temporal dynamics of the NADPH-response was different. Whereas the velocity of the increase in fluorescence was similar (0.079 s^{-1} *vs* 0.054 s^{-1} for KCN and NMDA, respectively), the decrease in fluorescence was one order of magnitude lower for KCN (0.0034 s^{-1} *vs* 0.023 s^{-1} for KCN and NMDA, respectively). As for NMDA stimulation, repeated injections of KCN had the same effect, *i.e.*, a transient increase in NAD(P)H-fluorescence (Figure 5b).

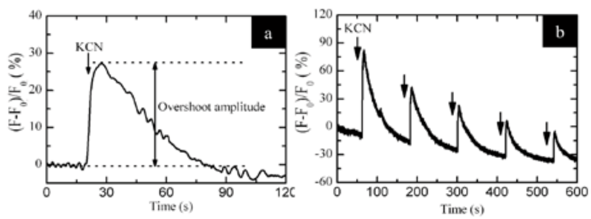

Figure 5. Effect of KCN on cellular NAD(P)H fluorescence. (a) The experiments were performed as described in Figure 2 except that 60 nl 0.5 M molar KCN was applied (final concentration 0.1 mM). (b) Repeated injection of KCN, each 70 nl as marked by arrows, was performed in the same way as described for (a). The age of the cells was 10 DIV.

The effect of KCN showed developmental changes (Figure 6). An increase in the overshoot amplitude was observed between 5 DIV to 7 DIV (19±0.8% to 24±0.9% P<0.001) followed by a plateau between 7 DIV and 15 DIV (25±1.2%) all P-values larger than 0.05). Between 16 and 25 DIV there is a steep decrease in amplitude down to 10% (P<0.0001 for DIV 15-18; P>0.9 for DIV 18-22, and P<0.004 for DIV 22-25).

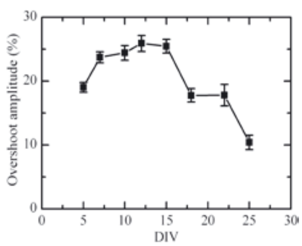

Figure 6. Dependence of the KCN effect on age of the cells. The experiments were performed as shown in Figure 5. The overshoot amplitude is plotted as a function of age of the cells. The percentage of responding cells was in every experiment 100%. Error bars represent the mean ± S.E.M of 24 to 50 cells.

3.5 NMDA-induced NAD(P)H responses during respiratory inhibition

Changes in NAD(P)H-fluorescence were used as a proxy for energy metabolism. However, these changes in fluorescence do not allow discrimination between glycolytic and mitochondrial contribution. In order to get estimates of the relative contributions of these two metabolic processes, mitochondrial respiration was blocked by addition of 2 mM KCN to the recording medium [8]. Cells were then incubated for 4 minutes in this solution. Following incubation in KCN, subsequent injection of 60 nl of 25 mM NMDA to the cells still invoked an increase in the cellular NAD(P)H-fluorescence indicating that the NMDA-effect is mainly due to stimulation of glycolysis. Similar to findings presented above (Figure 4), this stimulation exhibited developmental changes in the presence of KCN. NMDA induced overshoot amplitude in cells with and without KCN addition during the first 3 weeks *in vitro* (Figure 7).

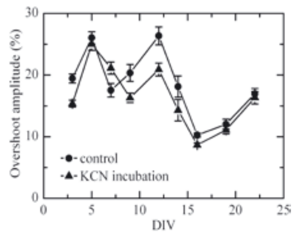

Figure 7. Effect of NMDA on energy metabolism of aerobic and anaerobic cell cultures. The cells were incubated in recording medium and 60 nl 25 mM of NMDA was injected while the NADH fluorescence was measured (circles). Subsequently, the recording medium was replaced by recording medium including 2 mM KCN. The cells were then incubated for 4 min with KCN before another NMDA injection (60 nl 25 mM) was done (triangles). The results show the normalized NMDA evoked overshoot amplitude, as defined in Figure 1. Error bars represent the mean ± S.E.M of 23 to 76 cells.

In both cases, we find two minima, where the differences between the minima and maxima are significant for both curves (P<0.01). The first minimum is located at 7 DIV (18±1.1) for cells without KCN and at 9 DIV (16±0.8) for cells with KCN. The second minimum is centered at 16 DIV for both cell types (9±0.3). Second, we have compared the values for each day of the 2 curves. The results show that the differences between these two curves from DIVs 6 to 16 are small but significant (P<0.02) except for DIV 14 (P>0.1). At all other DIVs the differences between the curved of the control and KCN inhibited cells are not significant (P>0.1).

4. Discussion

4.1 NMDA-evoked stimulation of cellular NAD(P)H-fluorescence

This study clearly demonstrates that hippocampal cell cultures from newborn rats respond to a local NMDA stimulus with an increased activity of energy metabolism, as indicated by changes in cellular NAD(P)

H fluorescence. The NAD(P)H fluorescence in brain slices from adult rats in response to electrical stimulation and/or receptor activation has been studied previously [7,13,14]. In these studies biphasic responses of the fluorescence signal have been found, consisting of an initial dip component (decrease of fluorescence) and a subsequent overshoot component. We found a different behavior when using cell cultures from the hippocampus of newborn rats instead of brain slices from adult rats. We observed only a transient increase in NAD(P)H fluorescence (*i.e*. the overshoot component). These discrepancies might be due to developmental status of each tissue preparation. Cells from newborn rats were still immature (even at 22 DIV) compared to those in adult brain slices. Furthermore, the development of neuronal network connectivity, including the expression of glutamatergic receptors, is continuing during the cultivation period. Finally, acute brain slices from adult rats likely have tighter coupling between neuronal and glial cells than present in our dissociated cultures from neonatal rats. On the other hand, the two-phase behavior is not seen in all hippocampal subregions, since it has been shown that some hippocampal regions only display the initial dip component [7].

The transient nature of the NMDA effect in our experiments is probably due to diffusion of NMDA out of the area of observation. The loss of stimulation leads to a reoxidation of NAD(P)H *via* the basic turnover of the energy metabolism. Experiments with multiple stimulations support this view. The fact that the magnitude of this fluorescence change decrease with every further injection can be explained by the gradual increase of NMDA with preceding injection.

4.2 Developmental changes of the energy metabolism

NMDA-evoked stimulation of the energy metabolism at different growth times, from 3 DIV to 26 DIV, likely signifies important developmental changes, as both changes in the overshoot amplitude as well as in the percentage of responding cells followed similar developmental profiles. The biphasic profile of overshoot amplitudes may indicate a “silencing” of metabolic activation during specific developmental windows, in which neuronal and synaptic reorganization takes place. Such windows have been detected for other neuronal parameters during hippocampal development. For example, Kudryashov *et al.* [15,16] showed that population spike (PS) amplitude increased until postnatal day (PND) 19, but decreased thereafter to a minimum between PND 21-PND 24, before it finally increased to adult levels. A study by Collard *et al.* [17], measuring glutamate release after K^+-depolarization in hippocampal synaptosomes, observed a dampened K^+-evoked glutamate response at PND 7 and between PND 14 and 16, after which the glutamate response increased again to adult levels. A recent study by Gruss *et al.* [18] found that emotional reinforcement of late-LTP was specifically prevented in adolescent rats, which had been exposed to maternal separation stress at PND 9, but not at other time points.

Kudryashov *et al.* and Collard *et al.* argue that these minima in either PS amplitude or glutamate release result from maturation processes of the early postnatal brain. In this study, it is unlikely that the developmental changes observed in our cultures are due to LTP induced changes of network connectivity. Accordingly, we tested whether the observed developmental patterns are the sole result of changes in energy metabolism, *i.e*. whether they are independent of activation by NMDA. Not surprisingly, energy flow, as estimated by cyanide induced accumulation of NAD(P)H, exhibits developmental changes. However, this change follows a bell-shaped curve during the first three weeks *in vitro* and is thus different from the biphasic patterns obtained by NMDA application. Hence, the growth dependent changes in energy metabolism alone may participate but cannot be the exclusive reason for occurrence of the biphasic response measured as NMDA triggered NAD(P)H-fluorescence change. From this we conclude that activation of the NMDA-receptor, either in neurons, glia cells or both, is required to produce the observed biphasic patterns in the energy metabolism. Moreover, the similarity of the developmental patterns of the activity of neurons [15-17] and the NMDA-evoked metabolic responses in our cultures indicate that both phenomena might reflect reorganization of neurons and their synaptic circuits. Consequently, we suggest that the decrease of neuronal connections at 7 DIV and 16 DIV could account for the decrease in NMDA-induced NADPH-response due to dampened neurometabolic coupling between neurons and glia cells, thereby reducing metabolic response towards NMDA.

4.3 NMDA-receptor activation in the presence of KCN

While NAD(P)H fluorescence is an appropriate proxy for energy metabolism, it does not allow the distinction between aerobic (*i.e*. mitochondrial) and anaerobic (*i.e*. glycolytic), energy production to be made. In general, NAD(P)H originates mainly from the mitochondrial energy metabolism: however, other energy sources are also known to be important. For example, Yamane *et al.* [19] have shown that anaerobic metabolism is important for neuronal function. Kasischke *et al.* [8] reported NAD(P)H-fluorescence changes as a result of glycolytic activity in glia cells, rather than in neurons. Kahlert and Reiser [20] found that glycolytic and (to a lesser extent) mitochondrial ATP was required for loading of intracellular Ca^{2+} stores of hippocampal astrocytes.

Therefore, to determine the source of the NAD(P)H signals in our cell cultures, we applied NMDA to cells whose respiration had been blocked by KCN. Under this condition, there is only a slight reduction of the NMDA-evoked overshoot amplitude. Moreover, the biphasic pattern with the two minima around DIV 7 and DIV 16 was still observed. As addition of cyanide completely blocks energy production by mitochondria, these results indicate that the observed changes in the NAD(P)H signals result mainly from glycolysis. This conclusion is supported by an earlier study from Zhan *et al.* [21], in which they found an NMDA-induced intracellular acidification in hippocampal slices. They attributed this NMDA-induced acid shift to glycolysis because the shift was reduced in the presence of the glycolytic inhibitor fluoride, and when glucose was substituted by pyruvate. Further support for this hypothesis regarding neurometabolic coupling [4,5], comes from the results of Shuttleworth *et al.* [7], who found that the activity of plasma membrane ATPase significantly contributed to stimulus evoked NAD(P)H signals in brain slices. From their results, they concluded that mitochondrial functions are not required for the NAD(P)H transients. Our findings support this, since NMDA can evoke the NAD(P)H transient in cyanide inhibited cell cultures.

Another interesting conclusion can be drawn, when comparing the developmental changes of the NMDA-evoked overshoot amplitude with those of the KCN-evoked overshoot amplitude. While the NMDA-evoked overshoot amplitude starts to increase during the third week *in vitro*, the KCN-evoked overshoot amplitude shows the opposite behavior, that is, it declines to minimal values. If we take the KCN-evoked signal as an indicator for the mitochondrial activity, these data indicate that the glycolytic energy production increases during the development of neuronal networks with respect to the mitochondrial one.

Taken together, our results demonstrate that energy metabolism of hippocampal cell cultures undergoes major changes during development of neuronal networks, fostering the idea of energy supported network formation. The observed changes appear to be due to glycolytic pathways, although additional pathways which may affect the efficiency of the neurometabolic coupling, can not be excluded.

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