Neuron survival in vitro is more influenced by the developmental age of the cells than by glucose condition

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Abstract The objective of this study was to determine whether the sensitivity to varying glucose conditions differs for the peripheral and central nervous system neurons at different developmental stages. Ventral horn neurons (VHN) and dorsal root ganglion neurons (DRG) from rats of different postnatal ages were exposed to glucose-free or glucose-rich culture conditions. Following 24 h at those conditions, the number of protein gene product 9.5 positive (PGP⁺) DRG neurons and choline acetyltransferase positive (ChAT⁺) VHN were counted and their neurite lengths and soma diameters were measured. For both DRG and VHN, the highest number of cells with and without neurite outgrowth was seen when cells from postnatal day 4 donors were cultured, while the lowest cell numbers were when neurons were from donors early after birth and grown under glucose-free conditions. The length of the neurites and the soma diameter for VHN were not affected by either glucose level or age. DRG neurons, however, exhibited the shortest neurites and smallest soma diameter when neurons were obtained and cultured early after birth. Our results indicate that survival of neurons in vitro is more influenced by the developmental stage than by glucose concentrations.

Keywords Age · DRG · Glucose · In vitro · Neurite outgrowth · Ventral horn neuron

Introduction

Hypoglycemia may damage neurons in both the central nervous system (CNS) and the peripheral nervous system (PNS), although in the PNS hypoglycemia preferentially damages myelinated motor axons (Jamali and Mohseni 2006; Mohseni et al. 2000; Sima et al. 1989). Sensory nerve fibers may be less affected (Mohseni et al. 2000) while dorsal root ganglion neurons (DRG) are not affected at all (Mohseni 2000) or only slightly influenced (Sima et al. 1989). In the CNS, short-term hypoglycemia causes neuronal death in the brain (Agardh et al. 1980, 1981; Auer et al. 1984a, b, 1985a, b; reviewed by Mohseni 2001), implying that the CNS neurons are more sensitive to low glucose than those of the PNS.

It has been reported that peripheral nerves from younger rats are more vulnerable to prolonged severe hypoglycemia than those from older animals (Yasaki and Dyck 1990). In the brain, Kim et al. (2005) observed more cell injury in hypoglycemic mice at postnatal day 7 (P7) than at P21. In addition, hippocampal slices prepared from P3 and P7 showed more neuronal injury than those taken from P14 and P21 mice (Kim et al. 2005). On the other hand, Fern et al. (1998) observed irreversible loss of function in optic nerves taken from adult rats after 60 min of glucose

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deprivation, while those taken from rats aged P5–P20 showed little resistance and nerves taken at age $\leq$ P4 were highly resistant. In the brain, consumption of glucose is altered (Willis et al. 2002) and glucose transporter 3 (Glut3) expression is reduced with increasing neuronal age (Fattoretti et al. 2001). Patel and Brewer (2003) found that the rate of glucose uptake in cultured embryonic neurons was threefold greater than in neurons taken from middle-aged and old donor rats. Thus, it is possible that the age of neurons may have an effect on whether those cells will recover or become overwhelmed by glucose deprivation. In this study we addressed the question whether the sensitivity of PNS and CNS neurons to glucose condition differs at different developmental stages.

Materials and methods

The experimental protocols were approved by the Ethics Committee for Animal Experimentation in Southeast Sweden. The rats were time-mated at B & K Universal AB, Sollentuna, Sweden. Each experiment was repeated at least three times, and data were quantified on coded slides. Unless otherwise indicated, the chemicals used in this study were purchased from Sigma–Aldrich (Sweden).

Ventral horn neurons

On the day after birth (P1) or postnatal day 4 (P4), Sprague–Dawley (SD) rat pups were decapitated and dipped in 70% ethanol. The vertebral column was quickly removed, and the ventral parts of the cord were dissected out and placed in cold PBS containing 15 mM glucose. The cords were chopped and trypsinized for 15 min in 2 mL of preheated (37 °C) 0.05% trypsin. From the end of that treatment until time for incubation in the culture media, the cells and solutions were kept cold (10 °C). The chopped samples were moved to 2 mL of L15 medium (Leibovitz's; Invitrogen) supplemented with 100 μ L of DNase, 100 μ L of 4% bovin serum albumin (BSA), and 200 μ L of fetal calf serum (FCS). The suspensions were shaken manually for 3 min and then allowed to settle for a few minutes. Thereafter, the supernatant was placed in 2 mL of L15 and transferred to a tube containing 1 mL of 4% BSA,

and the sample was centrifuged for 10 min (300 $\times$ g). The pellet was triturated in 4 mL of L15, and the cell suspensions were layered onto 2 mL of 10.4% Optiprep and then centrifuged for 15 min (513 $\times$ g). The resulting cell layer was added to 6 mL of L15 and subsequently layered onto 1 mL of 4% BSA, and the sample was centrifuged for 15 min (300 $\times$ g). The cells were added to pre-incubated (37 °C) L15 supplemented with 10 mM NaHCO₃, 0.1 mM putrescine, 5 μ g/mL insulin, 1 nM progesterone, 1% penicillin–streptomycin (PEST), 1 ng/mL brain derived neurotrophic factor (BDNF), 20 ng/mL IGF, 2.5 μ g/mL transferin, 2.5 μ g/mL sodium selenite, and 0 or 15 mM glucose. The cells were incubated for 24 h (37 °C, 5% CO₂, 95% humidity) on poly-L-ornithine (0.01%) and laminin (10 μ g/mL) pre-coated culturing slides (4,000 cells per chamber). Thereafter, the cells were washed with PBS and fixed in 4% paraformaldehyde (PFA, 15 min), and then incubated with rabbit antibodies against choline acetyltransferase (ChAT, 1:2000; Chemicon, England) in PBS containing 0.25% BSA, 0.25% Triton X-100, and 0.25% donkey normal serum followed by Cy3-conjugated anti-rabbit IgG (1:600 for 1 h at RT; Jackson, Sweden). The control slides were not incubated with primary antibodies. The specificity of primary antibodies was examined once by replacing them with normal rabbit serum. None of these negative controls showed specific immunoreactivity.

The slides were mounted with aqueous mounting medium (Dako, Denmark). The total number of ChAT⁺ cells with or without neurite outgrowth was counted in each chamber using a light microscope ($\times$ 100). Measurements of neurite length and soma diameter were performed on digital micrographs ($\times$ 400) using Easy Image Measurements[®] 2000 software (Tekno Optik, Stockholm, Sweden). Any outgrowth projecting $\geq$ 20 μ m from the soma of a ChAT⁺ cell was defined as a neurite. The method we used for isolation of VHN was adapted from the protocol for isolation and culture of mouse motor neurons developed by Anderson et al. (2004).

Dorsal root ganglion cells

On the day of birth (P0), P4 or P6 rat pups were decapitated and dipped in 70% ethanol. The DRGs at vertebrae L4–L5 were removed and placed in cold Hank's solution. The ganglia were then incubated in

0.12% collagenase in PBS (25 min, 37 °C) followed by 0.25% trypsin in Hank's solution. Thereafter, the tissue was triturated in Dulbecco's modified Eagle medium (DMEM) supplemented with 6 mM glucose, 10% fetal bovine serum (FBS), and 1% PEST and then centrifuged (240×g; 10 min). The cells were pre-plated in DMEM (1 h, 37 °C, 5% CO₂). The neurons were moved to a new dish, pre-plated (60–75 min), and then centrifuged for 10 min. The pellet was placed in 0, 6, or 15 mM glucose-DMEM solution supplemented with 50 ng/mL nerve growth factor (NGF), FBS and PEST. Neurons were plated at a density of 5,000 per chamber on poly-L-lysine-coated culture slides. After 24 h, the neurons were fixed in 4% PFA (20 min, 22 °C) and washed in PBS. The cells were incubated over night with rabbit antibodies against protein gene product 9.5 (PGP 9.5, 1:2000; Chemicon, UK) as neuronal marker, in PBS as mentioned above. After rinsing in PBS, cells were incubated with Cy3-conjugated donkey anti-rabbit IgG (1:200 at 20 °C for 30 min; Jackson). The control slides and the specificity of primary antibodies were tested as described for VHN. PGP 9.5⁺ cells with and without neurite outgrowth, were counted and neurite length and soma diameter were measured as previously mentioned. Any projections from neurons that were found to be $\geq 100 \mu\text{m}$ in length were considered to be neurites.

Statistics

Statistical analyses were performed using Student's two-sample *t*-test, assuming non-equal variances with Minitab software. We considered $P \leq 0.05$ to be significant.

Results

Ventral horn neurons

ChAT⁺ neurons, with or without neurite outgrowth and with mainly round or oval perikarya were observed (Fig. 1a). Most of the neurite-bearing neurons occurred singly or in sparsely populated groups, while those without neurite outgrowth appeared in clusters. The highest number of neurons per chamber was found for P4 cells cultured under glucose-free or glucose-rich conditions ($P < 0.001$; Table 1). The highest mean number of cells with neuritic processes was also seen for P4 neurons cultured in the absence or presence of glucose ($P \leq 0.01$; Table 1). At each particular donor-cell age the absence of glucose did not affect the number of cells with neurites. Most of the neurons that did develop neurites had only one primary projection, and the length of those processes was the same irrespective of glucose condition or age of the donor rats (Table 1). No differences were found in soma diameter of ChAT⁺ cells; The mean soma diameter ($\pm$ SEM) for P1 neurons cultured in glucose-free medium ($9.2 \pm 0.5 \mu\text{m}$; max $25.5 \mu\text{m}$) or in the presence of 15 mM glucose ($11.0 \pm 0.5 \mu\text{m}$; max $23.0 \mu\text{m}$) was the same as those for P4 neurons cultured in glucose-free ($12.2 \pm 0.6 \mu\text{m}$; max $22.4 \mu\text{m}$) or in glucose-rich medium ($13.3 \pm 0.7 \mu\text{m}$; max $27.1 \mu\text{m}$). Only a few non-neuronal cells were observed on some slides.

Dorsal root ganglion cells

The PGP 9.5⁺ cells had rounded or oval perikarya of varying size. Most of the cells cultured on P0 (i.e. the

Fig. 1 Representative fluorescence photomicrographs of ChAT-labeled neurons from the ventral horn of the spinal cord (a), and PGP 9.5-labeled DRG neurons (b). The bars in the figures indicate 10 μm (a) and 100 μm (b)

Table 1 Mean total number of ventral horn motor neurons, mean number and proportion (%) of neurons with neurites $\geq 20 \mu\text{m}$ long, and the length of neurites/neuron cultured in medium containing 0 or 15 mM glucose for 24 h

Age	Glucose concentration (mM)	Total no. of cells/chamber	Cells with neurites/chamber (%)	Neurite length/neuron (μm)
P1	0	47 $\pm$ 6	4 $\pm$ 0.9 (8.5%)	38 $\pm$ 3
	15	44 $\pm$ 7	5 $\pm$ 2 (11.4%)	29 $\pm$ 3
P4	0	74 $\pm$ 9 ^a	8 $\pm$ 2 ^c (10.8%)	32 $\pm$ 3
	15	60 $\pm$ 5 ^b	10 $\pm$ 0.3 ^d (16.7%)	34 $\pm$ 2

P postnatal day

Values are expressed as mean $\pm$ SEM of three sets of experiments^{a,b} ($P < 0.001$) and ^{c,d} ($P \leq 0.01$) show significance differences compared with corresponding values for P1

day of birth) had only one small neurite regardless of the glucose concentration. Most of the P4 and P6 cells developed two neurites with some bifurcation (Fig. 1b) or short neurites interwoven around the cell like a basket. In a few cases, we observed networks of neurites. DRG neurons, cultured from P0 rats, were most sensitive to the glucose conditions. At this age, the mean total number of PGP 9.5⁺ neurons per culture-slide chamber was significantly higher in 6 mM glucose than in 0 mM and 15 mM glucose ($P < 0.001$, Table 2). By comparison, the mean number of P0 neurons per chamber in glucose-free medium was almost half of the mean found for P4 and P6 neurons ($P < 0.001$; Table 2). The sensitivity of DRG neurons to glucose level diminished with increasing age of the donor rats and was not observed in P4 and P6 neurons. The highest number of neurons as well as the highest number of neurons which developed neurites were seen when cells from P4 donors were cultured. In the presence of 6 mM glucose, the number of cells per chamber was higher for P4 compared to P0 ($P = 0.003$) and P6 (non-significant) neurons. The same pattern was seen in the presence of 15 mM glucose i.e. the number of P4 neurons was greater than the number of P0 ($P < 0.001$) and P6 ($P < 0.03$) neurons (Table 2). The number of neurons that developed neurites $\geq 100 \mu\text{m}$ was also higher when neurons from P4 donors were cultured in the presence of 6 or 15 mM glucose (vs. P0 and P6, $P < 0.001$; Table 2). The length of neurites was affected by age but not by

Table 2 Mean total number of dorsal root ganglion cells, mean number and proportion (%) of neurons with neurites $\geq 100 \mu\text{m}$, and neurite length/neuron cultured in medium containing 0, 6 or 15 mM glucose

Age	Glucose concentration (mM)	Total no. of cells/chamber	Cells with neurites/chamber (%)	Neurite length/neuron (μm)
P0	0	76 $\pm$ 40 ^a	35 $\pm$ 21 (46%)	276 $\pm$ 32
	6	132 $\pm$ 25 ^b	51 $\pm$ 4 (38.6%)	266 $\pm$ 35
	15	90 $\pm$ 31	31 $\pm$ 20 (34.4%)	232 $\pm$ 15
P4	0	158 $\pm$ 43	74 $\pm$ 17 (46.8%)	273 $\pm$ 32
	6	192 $\pm$ 64 ^c	105 $\pm$ 33 ^e (54.7%)	263 $\pm$ 43
	15	134 $\pm$ 25 ^d	62 $\pm$ 4 ^f (46.3%)	301 $\pm$ 31 ^g
P6	0	139 $\pm$ 59	62 $\pm$ 30 (44.6%)	285 $\pm$ 28 ^h
	6	109 $\pm$ 39	47 $\pm$ 16 (43.1%)	229 $\pm$ 20
	15	107 $\pm$ 47	44 $\pm$ 25 (41.1%)	261 $\pm$ 39 ⁱ

P postnatal day

Values are expressed as mean $\pm$ SEM of $\geq$ three sets of experiments

^a $P < 0.001$ (vs. P4 or P6, 0 mM glucose); ^b $P < 0.001$ (vs. P0, 0 or 15 mM glucose); ^c $P = 0.003$ (vs. P0, 6 mM glucose); ^d $P < 0.001$ (vs. P0) and $P < 0.03$ (vs. P6), 15 mM glucose; ^e $P < 0.001$ (vs. P0 and P6, 6 mM glucose); ^f $P < 0.001$ (vs. P0 and P6, 15 mM glucose); ^g $P < 0.001$ (vs. P0, 15 mM glucose); ^h $P < 0.04$ (vs. P0, 0 mM glucose); ⁱ $P < 0.02$ (vs. P0, 15 mM glucose)

glucose availability (Table 2). In the absence of glucose the P6 neurons had longer neurites than P0 neurons ($P < 0.04$), and in the presence of 15 mM glucose both P4 ($P < 0.001$) and P6 ($P < 0.02$) neurons had longer neurites than P0 neurons (Table 2). Half of the P0 neurons were small with a soma diameter $< 30 \mu\text{m}$ and the other half were medium-sized with a diameter between 30 and $40 \mu\text{m}$; only a few neurons were large in diameter ($\geq 40 \mu\text{m}$; Fig. 2). The mean soma diameter (mean $\pm$ SEM) of P0 neurons cultured in medium containing 0 mM (30 ± 1.2), 6 mM (32.3 ± 1.1) or 15 mM (31 ± 1.1) glucose were significantly smaller than those of P4 neurons (0 mM, 38.3 ± 1.3 ; 6 mM, 37.6 ± 1.4 ; 15 mM, 34.3 ± 1.0 , $P < 0.05$) and P6 neurons (0 mM, 41.1 ± 1.4 ; 6 mM, 38.5 ± 1.1 ; 15 mM, 38.3 ± 1.2) cultured in medium with the corresponding level of glucose. The significance level (P) was < 0.001 if not otherwise mentioned. Only a few non-neuronal cells were observed on some slides.

Fig. 2 Histogram shows size distribution of soma diameter of DRG neurons cultured from pups at the day of birth (a), postnatal day 4 (b) and 6 (c). The mean soma diameter of P0 neurons cultured in medium containing 0, 6 or 15 mM glucose were significantly smaller than those of P4 ($P < 0.05$) and P6 ($P < 0.001$) neurons cultured in medium with corresponding glucose concentration

Discussion

Here we studied whether the sensitivity of PNS and CNS neurons to various glucose conditions differs at different developmental stages. Our results from experiments with VHN and DRG neurons showed that age of the donors affects the number of surviving neurons and the number of neurons which developed neurites. In the case of DRG neurons, the lowest number of surviving cells was observed when P0 neurons were cultured in glucose-free condition. In addition, irrespective of glucose conditions, almost all P0 neurons were small or medium sized ($<40 \mu\text{m}$), and the largest neurons with soma diameter $\geq 40 \mu\text{m}$ were rarely observed in these cultures (Fig. 2). These results indicate that neurons taken from donors early after birth were more sensitive to glucose deficiency and the in vitro condition than those removed from older donors. The length of neurites was not affected either by age or glucose

level in VHN, but was influenced by age in DRG neurons.

To the best of our knowledge, our work is the first to consider the effects of glucose deficiency on VH and DRG neurons at different developmental ages. In the case of VHN, the number of studies conducted in vitro is very limited due to the poor survival of these cells in culture. It might be possible to improve their ability to stay alive in a number of ways, for example, by using pups instead of embryos, since the natural death of motor neurons seen during embryogenesis (Yamamoto and Henderson 1999) does not occur at postnatal ages (Oppenheim 1986). Accordingly, we used rat pups in our experiments on VHN and DRG neurons. Another plausible method seems to be the use of proteins of the neurotrophin family, since 1 ng/mL BDNF in the culture medium has been reported to enhance the survival of rat motor neurons by 60% (Hughes et al. 1993). Therefore, we used the mentioned concentration of BDNF to support the VHN in our experiments. We found no difference in survival rate between glucose-deficient and glucose-rich conditions for P1 VHN. However, P4 neurons did exhibit higher survival rates and more cells with neurites in relation to age of seeded cells but not to glucose availability. In the absence of glucose, the mean number of neurons per chamber was significantly higher for cells from P4 donors than those from P1 donors, and the same pattern was seen in the presence of 15 mM glucose (non-significant). This may indicate either that neurons cultured from rats at birth are more sensitive to glucose deficiency and/or in vitro conditions, or that the natural cell death program is still active after birth. The absence of large neurons in the VHN cultures indicates that these neurons were most sensitive to in vitro condition. Overall, our findings indicate that the survival of rat VHN and the ability of those cells to develop neurites are not affected negatively by 24 h of glucose deficiency in vitro, that the VHN are more extensively influenced by age-associated factors than by glucose availability, and the largest VHN are most sensitive to in vitro condition.

DRG neurons have been cultured in a range of glucose concentrations in different studies, but unfortunately, there is no common definition of normal, low, or high glucose levels for evaluating these cells in vitro. Some investigators have considered 10–30 mM glucose to be normal/optimal and 45 mM as a high concentration (Russell et al. 1999, 2002; Vincent et al.

2004). On the other hand, Sango et al. (2002) used 30 and 10 mM glucose to represent high level and control conditions, respectively. Commercially available culture media usually contain 25 mM glucose. The different definitions of a normal level of glucose for DRG neurons in vitro and the disparities in experimental design make it difficult to compare the results of different studies. In the current experiments, we used 6 or 15 mM glucose in the culture medium for comparison with glucose-deficient conditions. It can be argued that these concentrations represent low glucose levels and, thus, the lack of divergence in survival of neurons (P4 and P6) at different glucose level might result from limited glucose availability during the experiments. However, we argue that this was not the case, since the normal blood glucose concentration in rats is around 5 mM, and hence the tissues does not have access to higher levels. We found that the P0 DRG neurons were most sensitive to glucose deficiency, because under those conditions they exhibited a survival rate that was only half that observed upon exposure to 6 mM glucose and not quite two-thirds of that seen in the presence of 15 mM glucose. However, this pattern was not found when P4 or P6 DRG neurons were cultured in the absence of glucose, which suggests that sensitivity to glucose deficiency diminishes with age. In addition, P0 DRG neurons showed the lowest survival, even in the presence of 15 mM glucose, which implies that the survival rate is affected by age. The length of neurites was also influenced by age but not by glucose concentration; more precisely, for each particular age of the cell donors, the length of neurites grown in the presence of 0, 6 or 15 mM glucose was the same. Nevertheless, differences were found between the neurons from rats of different ages i.e. in the absence of glucose P6 neurons had significantly longer neurites than P0 neurons, and in the presence of 15 mM glucose, both P4 and P6 neurons had significantly longer neurites than P0 neurons (Table 2). Regardless of glucose level or age of the donors, such projections grew to 230–300 μm after 24 h in our study, which is about half the length of the processes observed by other researchers on DRG neurons cultured for 48 h (Russell et al. 1999). This finding indicates that DRG neurons in vitro can increase the length of their neurites by about 10–15 μm per hour, and that ability is not affected by the concentration of glucose. Further, we did observe only few neurons with a soma

diameter larger than 40 μm when P0 neurons were cultured in different glucose concentrations. From the results of soma diameter we cannot establish the functional properties of DRG neurons in our study. Lawson et al. (1993) showed that most small neurons are substance P-like immunoreactive (SP-LI) neurons, and measurements of conduction velocity showed that about half of the C-fibre neurons and 10% of A δ -neurons but no A α / β -neurons were SP-LI. Calcitonine gene-related peptide immunoreactivity was observed in small, medium-sized and large neurons with conduction velocity in the C, A δ or A α / β ranges. For coming closer to an identification of neurons functional properties, the information of soma diameter should be combined with information from conduction velocity, neuropeptides and amount of neurofilaments in a particular neuron. Taken together, our results suggest that neither the survival of DRG neurons nor the capacity of these cells to develop neurites is influenced by glucose deficiency in vitro. Only neurons taken from donors early after birth showed some sensitivity to glucose deficiency and to in vitro conditions. As seen in ventral motor neurons, it is plausible that DRG neurons in vitro are more profoundly affected by age-dependent factors than by the lack of glucose.

In conclusion, the results of our study suggest that the survival of VHN and DRG neurons in vitro may be more extensively affected by the developmental stage of the seeding cells than by glucose deficiency. Our findings also indicate that the effect of glucose-free conditions on neurons in vitro does not mimic the situation in vivo, and thus the interpretations of the in vitro data cannot be regarded as fully representative of the circumstances in vivo. More research is needed to understand the mechanism that protects neurons in vitro from the harmful effects of glucose deficiency.

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