The R6/2 transgenic mouse model of Huntington’s disease develops diabetes due to deficient β-cell mass and exocytosis

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Diabetes frequently develops in Huntington’s disease (HD) patients and in transgenic mouse models of HD such as the R6/2 mouse. The underlying mechanisms have not been clarified. Elucidating the pathogenesis of diabetes in HD would improve our understanding of the molecular mechanisms involved in HD neuropathology. With this aim, we examined our colony of R6/2 mice with respect to glucose homeostasis and islet function. At week 12, corresponding to end-stage HD, R6/2 mice were hyperglycemic and hypoinsulinemic and failed to release insulin in an intravenous glucose tolerance test. In vitro, basal and glucose-stimulated insulin secretion was markedly reduced. Islet nuclear huntingtin inclusions increased dramatically over time, predominantly in β-cells. β-cell mass failed to increase normally with age in R6/2 mice. Hence, at week 12, β-cell mass and pancreatic insulin content in R6/2 mice were 35 ± 5 and 16 ± 3% of that in wild-type mice, respectively. The normally occurring replicating cells were largely absent in R6/2 islets, while no abnormal cell death could be detected. Single cell patch-clamp experiments revealed unaltered electrical activity in R6/2 β-cells. However, exocytosis was virtually abolished in β- but not in α-cells. The blunting of exocytosis could be attributed to a 96% reduction in the number of insulin-containing secretory vesicles. Thus, diabetes in R6/2 mice is caused by a combination of deficient β-cell mass and disrupted exocytosis.

INTRODUCTION

Huntington’s disease (HD) is a fatal autosomal dominant neurodegenerative disease. The prevalence of HD is four to seven in 100 000 and it typically develops in mid-life. The symptoms involve psychiatric, motor and cognitive disturbances, and weight loss. In addition, there is an increased incidence of diabetes mellitus in HD patients. Ten to 25% of HD patients exhibit impaired glucose homeostasis (1). Reductions in both insulin sensitivity and secretion are apparent.

HD is caused by an expansion of a CAG repeat in exon 1 of the huntingtin gene (2), which encodes a protein suggested to be associated with synaptic vesicles and microtubules in neurons (3,4). There is evidence that huntingtin directly and/or indirectly plays a role in vesicle trafficking and neurotransmission (5). It has also been shown that normal huntingtin is anti-apoptotic in brain cells (6–8) and protects from toxicity induced by the mutant protein (9,10).

A common feature of mutant huntingtin and other mutant proteins involved in CAG-repeat expansion diseases is a tendency to form insoluble aggregates that accumulate in the cytoplasm and nucleus of cells (11). In HD patients, intranuclear inclusions have been described in the striatum and cortex (12,13), whereas in mouse models of the disease inclusions appear to be more widely distributed and have been demonstrated also in peripheral tissues (14). Huntingtin depositions are viewed by many as key players in the pathogenetic process in basal ganglia neurons in HD (13,15). The mechanisms, however, whereby striatal and cortical neurons die in HD have not been resolved. Impaired energy metabolism (16), excitotoxicity (17) and oxidative stress (18) have been implicated. Huntingtin is expressed not only in neurons, but also in several other tissues.
including endocrine tissues such as pancreatic islets (19). Therefore, if the same fundamental cellular pathology, presumably caused by mutant huntingtin, occurs also in pancreatic β-cells, it may explain why β-cells become dysfunctional in HD patients and diabetes subsequently evolves. Conversely, examining this assumed fundamental cellular dysfunction may shed new light on the pathological processes occurring in neurons.

To facilitate the study of HD pathogenesis, several transgenic mouse models of HD have been created (20). The first model was the R6/2 mouse (21), which expresses exon 1 of the human HD gene, containing 150 CAG repeats. Consequently, the mice develop neurological symptoms that resemble many of those seen in HD. These include deficits of motor co-ordination, altered locomotor activity, impaired cognitive performance and seizures. Specific behavioral tests detect cognitive symptoms already around week 3–4 (22), whereas motor symptoms typically develop later. In most breeding colonies, the R6/2 mice die at week 12–14 for unknown reasons. At this stage, there is significant atrophy of the striatum and neocortex and widespread occurrence of neuronal intranuclear inclusions of mutant huntingtin. Although we recently demonstrated loss of a specific population of orexin-expressing neurons in the hypothalamus of R6/2 mice and HD patients (23), surprisingly little evidence for cell death in the brain of R6/2 mice has emerged (13,24–26).

The R6/2 mice also exhibit pathological changes in tissues outside the central nervous system (27,28). For instance, huntingtin inclusions appear in skeletal and cardiac muscles, medullary cells in the adrenal gland, hepatocytes and pancreatic islets (14). With respect to pancreatic islets, the inclusions may be involved in the development of hyperglycemia and insulin-deficient diabetes in R6/2 mice (27,29). Whether the reduced levels of islet and circulating insulin are associated with reduced β-cell mass has not been determined (29). It has been proposed that R6/2 mice develop diabetes due to interference with transcriptional regulation in islets (13,29). This has been suggested to be caused by the insoluble nuclear protein aggregates containing huntingtin. The fact that misfolded huntingtin forms intranuclear inclusions also in pancreatic β-cells (13,29) suggests that pathological changes in β-cells have features in common with those that take place in the nervous system in HD. Thus, unraveling these processes may also shed light on pathological processes in the brain of R6/2 mice. Moreover, glucose homeostasis and islet function may be readily accessible parameters that can be monitored as measures of the progress of HD and to assess the effects of possible therapeutic interventions effective also in the brain. For these reasons, we set out to elucidate the pathogenetic mechanisms underlying diabetes in R6/2 mice. We found that dual events lead to the development of diabetes in the R6/2 mice: (1) replication of β-cells was impaired in R6/2 islets, resulting in deficient β-cell mass, and (2) β-cells exhibited a dramatically decreased number of insulin-containing secretory vesicles accounting for an abolishment of exocytosis.

RESULTS

Expression of huntingtin in pancreatic islets

Nuclear inclusions presumably derived from huntingtin have previously been demonstrated in islets from R6/2 mice (14). However, expression of the native protein in islets has not been described. To study this, islets were isolated from wild-type mice, untreated Sprague-Dawley rats and clonal insulin-producing cells (832/13 cells), and analyzed with RT–PCR. As shown in Figure 1A, huntingtin mRNA was present in rodent islets as well as clonal β-cells. The presence of huntingtin protein in the clonal β-cells and islets was confirmed by western blotting (data not shown).

To elucidate cellular expression of huntingtin in the pancreas, immunocytochemistry was performed. However, because only a monoclonal antibody to huntingtin was at our disposal, immunocytochemistry for this antigen in mouse pancreatic sections was technically not possible, due to the binding of the mouse secondary antibody to immunoglobulins in the tissue. Instead, to obtain some information on cellular localization of huntingtin in rodent islets, we performed experiments in rat pancreatic sections (Fig. 1B); here, the majority of islet cells expressed huntingtin, implying that β-cells account for the larger part of huntingtin expression in islets.

Nuclear inclusions in pancreatic islets

The pathology in HD patients and R6/2 mice may be associated with the formation of nuclear inclusions derived from the mutant huntingtin. Therefore, we examined whether islet cells in our colony of R6/2 mice also exhibit the nuclear inclusions typical of HD (13). Double immunocytochemistry in pancreatic sections, using antibodies to insulin and an antibody to the mutant huntingtin protein, showed that islets in wild-type mice lacked huntingtin inclusions (Fig. 2A). In R6/2 mice, however, inclusions could be seen in the nuclei of 19% (86 cells out of 450, three different mice) of β-cells already at week 7 (Fig. 2B); at week 12, the frequency of the inclusions increased dramatically and occurred in a majority (>95%) of the β-cells (Fig. 2C). Huntingtin inclusions could not be detected either in glucagon-producing α-cells or in somatostatin-producing δ-cells at 7 weeks, although at 12 weeks, 24% (51/212 cells, three different mice) of the α-cells and 6% (6/97 cells, four different mice) of the δ-cells contained inclusions. Clearly, the β-cell is more prone to huntingtin deposition than the neighboring islet cell types.

Metabolic characterization of the R6/2 mice

Body weight was similar in R6/2 and wild-type mice up to 10 weeks of age after which it declined in R6/2 mice (data...
not shown). Plasma glucose levels rose progressively in R6/2 mice compared with wild-type mice and at 12 weeks, the majority of mice were hyperglycemic (Fig. 3). Serum glucose was 19.6 ± 1.59 versus 12.3 ± 0.84 mM in wild-type mice (n = 10, P < 0.001). Circulating insulin levels gradually decreased over time in R6/2 mice. In 12-week-old R6/2 mice, serum insulin was 0.89 ± 0.21 versus 1.45 ± 0.32 ng/ml in wild-type mice (n = 10, P = 0.002).

**Glucose tolerance test**

To further examine glucose homeostasis, the mice were subjected to a glucose tolerance test at week 11.5. Glucose disposal was retarded in the R6/2 mice (Fig. 4A); the area under curve (AUC) was increased by 25% (P = 0.035). Remarkably, during the test, the insulin response was virtually absent (Fig. 4B); AUC for R6/2 mice was 66% of that in wild-type mice (P = 0.015). Together with the increase in basal plasma glucose and reduced circulating insulin levels, the results demonstrate that R6/2 mice develop insulin-deficient diabetes.

**Insulin secretion in vitro**

To further clarify the secretory deficiency observed in vivo, we examined insulin secretion in vitro in islets isolated from the mice. At week 7, insulin secretion from R6/2 islets was similar to that from wild-type islets in response to both 2.8 and 16.7 mM glucose (Fig. 5A). In contrast, at week 12, insulin secretion in response to both 2.8 and 16.7 mM glucose was reduced 4-fold (P < 0.001) in the islets from the R6/2 mice compared with wild-type islets (Fig. 5B). We also examined insulin secretion in response to 35 mM KCl. KCl depolarizes the β-cell and provokes insulin secretion, which is a measure of β-cell function independent of metabolic regulation. Again, insulin secretion from R6/2 islets was impaired compared with that from wild-type islets at 12 week (2.5-fold reduction, P = 0.012; Fig. 5B).

**Morphological and immunochemical characterization of islets**

Pancreatic sections from R6/2 and wild-type mice were immunostained for insulin. At week 7, the appearance and size of β-cell mass in the two genotypes were indistinguishable. In contrast, at week 12, islets in R6/2 mice appeared hypotrophic compared with the wild-type mice (Fig. 2).

To quantify the apparent reduction in β-cell mass, an unbiased analysis using stereological morphometry was performed in sections immunostained for insulin and prepared for light microscopy. Thus, confirming the observations from immunofluorescence for insulin, there was no difference in β-cell mass at week 7, as reflected by the surface area in the sections covered by β-cells. However, at week 12, insulin-immunostained area in pancreatic sections from R6/2 mice was 55% of that in wild-type mice (P < 0.0001) (Fig. 6). Islet number was unaffected at both time points (data not shown).

At week 7, insulin content in islets was similar in both genotypes. In contrast, at week 12, there was a significant reduction in insulin content in islets from R6/2 mice (Table 2). Furthermore, tissue content of the islet hormones was measured. As shown in Table 1, there was a dramatic decrease in pancreatic insulin content as well as somatostatin content in 12-week-old R6/2 mice, whereas glucagon content remained unchanged.

**FluoroJade-staining and evaluation of neogenesis/regeneration**

FluoroJade (FJ) stains dying cells in the brain as well as in other tissues (30). This stain revealed no signs of cell death at either 7 or 12 weeks (data not shown). Another potential explanation for the deficient β-cell mass in R6/2 mice is a reduction in neogenesis and/or regeneration of islet cells. These events require replication of cells and therefore, we injected mice systemically with the nucleotide analog bromodeoxyuridine (BrdU) and examined its incorporation into nuclei of dividing cells. Remarkably, there was a 6-fold reduction in BrdU-positive cells/islet in R6/2 compared with...
wild-type mice (Fig. 7), implying that replication of islet cells is impaired.

Cell death at an ultrastructural level

At this point, cell death had been examined by markers at the light and fluorescence microscopic level. To extend this work, we examined the islets at the ultrastructural level, to see whether any signs of apoptosis or necrosis could be identified. Confirming our observations using morphometry, the islets in 12-week-old R6/2 mice were considerably smaller as assessed by electron microscopy. While α- and β-cells appeared normal in appearance and frequency, the number of β-cells was dramatically reduced. Despite this reduction, no signs of apoptosis (e.g. membrane blebbing, pyknotic nuclei) or necrosis were observed (Fig. 8). This agrees with our findings at the light microscopic level and suggests that an impairment of replication, as opposed to cell death, is the cause of the observed reduction in β-cell mass in R6/2 mice.

Age-dependent effects on single-cell exocytosis of insulin and glucagon in R6/2 mice

We next characterized the secretory defect observed in R6/2 mice at the level of the single β-cell (Fig. 9). Exocytosis was measured as increases in cell capacitance (ΔC) and was evoked by trains of ten 500 ms voltage-clamp depolarizations that activated the endogenous voltage-gated Ca^{2+} channels. In control β-cells, such stimulation evoked an average 430 ± 15 fF capacitance increase (n = 13; Fig. 9A). When analyzing the amplitude of exocytosis evoked by the respective pulses of the train stimulus (Fig. 9B), the exocytotic response to the first depolarization was the largest (108 ± 8 fF) and then decreased during the train stimulus. This pattern of exocytosis is consistent with the view that the β-cell possesses a pool of immediately releasable insulin granules that is emptied within ~1 s after elevation of cytosolic Ca^{2+} (32). When R6/2 mice were examined at 8 weeks of age, insulin exocytosis remained largely intact and overall responses averaged 415 ± 11 fF (n = 11; Fig. 9C). Interestingly, closer inspection of the responses to the individual pulses in the train stimulation revealed that the first depolarization evoked a mere 24 ± 7 fF ΔC (P < 6.22748 × 10^{-5} versus exocytosis evoked by the first pulse in control β-cells) and that substantial exocytosis was not evoked until the third depolarization. This disturbance in exocytotic kinetics is reminiscent of the ‘uncoupled’ type of exocytosis observed in β-cells in which the physical association between the L-type Ca_{v}1.2 channels and the insulin granules has been disrupted (32,33). This suggests that the insulin exocytotic apparatus is not optimally functional in 8-week-old R6/2 mice. Indeed, when β-cells were isolated from 12-week-old R6/2 mice, depolarization-evoked exocytosis was virtually abolished (total 33 ± 2 fF, n = 7; P < 1.55356 × 10^{-13} versus exocytosis in control mice and P < 8.8325 × 10^{-15} versus 8-week-old R6/2 mice). It was ascertained that these effects on exocytosis occurred without any noticeable change in train depolarization-associated Ca^{2+} influx. In addition, after the train stimulation, Ca^{2+} current–voltage relations were investigated in all cells over the voltage range −50 to +50 mV. Maximal Ca^{2+} influx was invariably observed at 0 mV and averaged 74 ± 12, 82 ± 9 and 85 ± 14 pA at this potential in β-cells from control, 8- and 12-week-old R6/2 mice, respectively (data not shown).
discovery of novel therapeutic targets in the brain. a deeper understanding of why the pancreatic islets fail in also reflect important cellular changes that occur in neurons homeostasis observed in HD patients. In addition, they could important feature of the polyglutamine disease. Indeed, the mouse (39). This suggests that the endocrine pathology is an Hyperglycemia has also been observed in the HD-N171-82Q glucose homeostasis and frank diabetes occur with a variable of glucose intolerance and diabetes in patients with Alzhei- DISCUSSION Figure 6. Insulin-immunostained area in islets from 7- and 12-week-old mice. We next measured glucagon exocytosis evoked by train depolarizations in single α-cells from R6/2 mice (Fig. 10). Depolarization-evoked glucagon exocytosis was on average more intense than in the β-cells, in excellent agreement with previous observations (34). In control α-cells, a train depolarization evoked a 950 ± 47 fF increase in cell capacitance (n = 4), which remained largely intact also in the R6/2 α-cells (770 ± 33 and 700 ± 82 fF, n = 7 and 3, in 8- and 12-week-old R6/2 α-cells, respectively).

Ultrastructural β-cell appearance

A possible explanation for the disruption of exocytosis is depletion of secretory vesicles. To explore this possibility, islets were examined at the ultrastructural level. Indeed, β-cells from 12-week-old R6/2 mice contained very few insulin secretory vesicles (7 ± 1.5 compared with wild-type 171 ± 12.5 n = 10; Fig 8). This finding agrees with our determination of islet content of insulin (Table 2), which was relatively lower than β-cell mass (16 ± 3 versus 35 ± 5%), thus indicating that deficient β-cell mass alone cannot account for the reduction of islet insulin content.

Table 1. Insulin, glucagon and somatostatin content in pancreatic extracts from 12-week-old wild-type and R6/2 mice (mean ± SEM were given; Student’s t-test was used to assess difference between the two groups)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Insulin (ng/mg)</th>
<th>Glucagon (ng/mg)</th>
<th>Somatostatin (ng/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>110.5 ± 6.5 n = 8</td>
<td>3.21 ± 0.47 n = 8</td>
<td>291 ± 34.1 n = 6</td>
</tr>
<tr>
<td>R6/2</td>
<td>17.3 ± 4.2* n = 6</td>
<td>2.55 ± 0.64 n = 6</td>
<td>31.2 ± 4.9* n = 6</td>
</tr>
</tbody>
</table>

*P < 0.001.

In our studies of R6/2 mice, plasma glucose levels increased over time. Moreover, in the glucose tolerance test at week 12, glucose disposal was markedly retarded and the ability of glucose to provoke insulin secretion virtually absent. Insulin secretion and content in islets were unaffected at week 7 in R6/2 mice compared to wild-type mice. At week 12, insulin content was increased in the wild-type mice, whereas it was decreased in the R6/2 mice. Concomitantly, both basal secretion and glucose-stimulated insulin secretion were impaired at week 12. These observations were further substantiated by morphometric analysis of islets. Again, at week 7, islet number and β-cell mass were similar in both genotypes. In wild-type mice, β-cell mass was doubled when the mice reached the age of 12 weeks. In contrast, R6/2 islets did not increase in size. Clearly, the insulin secretory deficiency in R6/2 islets is paralleled by a deficiency in β-cell mass and insulin content, suggesting a casual relationship.

Previous reports have described reductions in pancreatic levels of glucagon, insulin and insulin mRNA in R6/2 mice (26,28); islet appearance in these studies was described as normal. In our study, we do not observe a marked reduction in the pancreatic level of glucagon. In fact, α-cells do not appear to be affected to the same extent as β-cells by nuclear inclusions of huntingtin (α-cells in the 12-week-old R6/2 mouse display intranuclear inclusions to the same extent as β-cells at week 7), despite the fact that α- and β-cells share common endocrine features as well as embryonic background (40). The observed reduction in somatostatin levels could be a compensatory mechanism, due to low levels of insulin; the foremost function of somatostatin in islets is as a paracrine inhibitor of insulin release (41). δ-cells were virtually unaffected by huntingtin inclusions and our preliminary studies suggest that the number of δ-cells is unchanged (data not shown).

We examined the underlying cause of the reduction in β-cell mass in R6/2 mice by using markers for cell death and replication as well as ultrastructural studies by electron microscopy. Surprisingly, there were no indications of increased cell death in islets. Instead, a marked decrease in the replication of β-cells was observed. This implies that neogenesis and/or regeneration of islet cells is impaired. Although the replication rate of β-cells is normally low (42), a reduction will ultimately result in reduced β-cell mass and could explain the reduction in β-cell mass in R6/2 mice. We have recently shown that there is a significant reduction in cell proliferation in the hippocampus of 12-week-old R6/2 mice compared with their wild-type littermates (43). Another group has reported an impairment of hippocampal cell proliferation in the R6/1 HD mouse model (44). A failure of proliferation of fibroblasts derived from 12-week-old R6/2 mice has also

Neurodegenerative disorders are associated with endocrine abnormalities (35,36). Several studies show a high prevalence of glucose intolerance and diabetes in patients with Alzheimer’s disease, Parkinson’s disease, Friedreich’s ataxia and HD (1,37,38). Importantly, it has been shown that perturbed glucose homeostasis and frank diabetes occur with a variable penetrance in R6/2 mice (26–28).

Diabetes is not restricted to one type of transgenic HD mice. Hyperglycemia has also been observed in the HD-N171-82Q mouse (39). This suggests that the endocrine pathology is an important feature of the polyglutamine disease. Indeed, the pathogenetic mechanisms in the pancreatic islets of the mouse models may be relevant to the changes in glucose homeostasis observed in HD patients. In addition, they could also reflect important cellular changes that occur in neurons and that contribute to the neurological symptoms. Therefore, a deeper understanding of why the pancreatic islets fail in transgenic mouse models of HD could ultimately lead to the discovery of novel therapeutic targets in the brain.
been reported (45). These findings together suggest a general-
ized impairment of cell replication, which could play an
important pathogenic role in various tissues in R6/2 mice. It
could provide an explanation for the reduced cell number
and atrophy found in some brain regions in R6/2 mice,
while signs of cell death have been more difficult to identify
(13,22–25).

Despite the striking deficiency of β-cell mass (~35%
reduction), this in itself may not be sufficient to provoke diabe-
tes in the mouse (46). For this reason, we also tried to iden-
tify a functional impairment of β-cells in R6/2 mice. We found
that the electrical activity in R6/2 β-cells was intact. In con-
trast, exocytosis induced by membrane depolarization was
abolished in β-cells from 12-week-old R6/2 mice. This
agrees with the dramatic reduction in the number of secretory
vesicles in R6/2 β-cells, as observed by electron microscopy,
raising the possibility of a specific perturbation of the exocy-
totic process in these cells. Indeed, exocytosis in α-cells was
unaffected. This agrees with the finding that nuclear inclusions
of huntingtin appeared to a lesser extent in α-cells; conse-
quently, the ultrastructural appearance of these cells was
unchanged. In fact, perturbations of exocytosis have been
demonstrated in in vitro models of HD, e.g. impaired
expression of the exocytotic protein complexin II in PC12
cells expressing mutant huntingtin (47). In these experiments,
however, reduced numbers of granules were not reported.

In conclusion, we have identified two separate pathological
processes that are responsible for diabetes in R6/2 mice:
impaired β-cell replication, resulting in deficient β-cell mass,
and a loss of insulin-containing secretory granules, abrogating
stimulated hormone secretion. As discussed, previous reports
have described similar impairments in experimental models of
HD (47). This highlights the importance of elucidating pathol-
gies in endocrine cell systems in HD. Resolving the pathogenic
and molecular events leading to β-cell loss and β-cell dysfunc-
tion could potentially contribute to the understanding of neuro-
ological symptoms in HD and the fundamental pathogenesis
of the disease. Such efforts may also generate tools for improved
monitoration and treatment of this devastating disease.

MATERIALS AND METHODS

Animals and plasma analyses

The colony of R6/2 mice has been previously described in
detail (43,48). For the present experiments, male and female
littermate heterozygote R6/2 and wild-type mice were used.
Blood was obtained by retro-orbital bleeding from anes-
ethetized mice [midazolam 0.4 mg/mouse (Dormicum®,
Hoffman-La-Roche, Basel, Switzerland) and a combination
of fluanison (0.9 mg/mouse) and fentanyl (0.02 mg/mouse;
Hypnorm®, Janssen, Beerse, Belgium)]. Glucose was
measured using the glucose oxidase method (Thermo Trace,
Victoria, Australia). Plasma insulin levels were determined
by radioimmunoassay (RIA; Linco Research Inc., St. Louis,
MO, USA). The experiments were approved by the Regional
Animal Ethics Committee in Lund.

R6/2 mice in the terminal phase, where neurological symp-
toms have evolved, have difficulties to procure any food. For
this reason, it was unethical and harmful to the mice to subject
them to blood sampling under fasting conditions, which would
have demonstrated an even more exaggerated metabolic
difference between mutant and wild-type mice.

Glucose tolerance test

For the intravenous glucose tolerance test, d-glucose (1 mg/g)
was injected into the tail vein of anesthetized mice (see pre-
vious subsection). Plasma glucose and insulin levels were
determined, as described previously, in retro-orbital blood
samples collected at the indicated time points.

Isolation and batch incubation of islets

Islets were isolated by standard collagenase digestion and
handpicked under a stereo microscope. Batches of three
islets for each condition were kept in HEPES balanced salt
solution (HBSS; 114 mM NaCl, 4.7 mM KCl, 1.2 mM
KH₂PO₄, 1.16 mM MgSO₄, 20 mM HEPES, 2.5 mM CaCl₂,
25.5 mM NaHCO₃, 0.2% BSA; pH 7.2) containing 2.8 mM
glucose for 60 min in an incubator at 37°C. Then, three
islets at a time were transferred to a 96-well plate kept on
ice and containing 200 µL per well of the same buffer but
with the addition of the respective secretagogue. Following
transfer of all islets, the plate was again placed in an incubator
at 37°C. At 60 min, a sample from the buffer was removed for
the measurement of insulin by RIA (Linco Research Inc.).
Islet content of insulin was measured after acid/ethanol
extraction.

Reverse transcriptase–PCR analysis

mRNA was isolated from rat isolated islets, mouse isolated
islets and clonal insulinoma cells (832/13 cells) (49).
The reverse transcriptase (RT) reaction was performed using
Superscript premiﬁcation system (Life Technologies,
New York, NY, USA) according to the manufacturers protocol. The synthesized first-strand cDNA reactions were stored at −80°C. A 1 μl of the cDNA-containing solution was used for the PCR reaction (reaction volume 20 μl) in 10 × PCR buffer containing 1.5 mM MgCl₂, 200 μM dNTP and 1 unit Taq enzyme (PCR reagents were purchased from Promega, Madison, WI, USA) with specific primers towards a 519 bp long fragment of mouse huntingtin. The primers for the detection of huntingtin cDNAs were 5'-TCGTACCACCATTTGTTTTTCA-3' and 5'-CCTTTGGCCTATGAGATCTGGATGTG-3'. Amplification was performed under the following conditions: denaturation at 94°C for 1 min, annealing at 56°C for 1 min and extension at 72°C for 2 min for a total 45 cycles.

Immunocytochemistry and morphometry

Pancreatic tissue was prepared for immunocytochemistry as previously described (50) and sections were cut on a cryostat (10 μm). An affinity-purified rabbit antibody to insulin (diluted 1:1280; code 9003; Euro-Diagnostica, Malmö, Sweden), a mouse anti-huntingtin protein monoclonal antibody (diluted 1:500, MAB 2166) and a mouse polyclonal antibody recognizing huntingtin inclusions (EM48 1:400 Chemicon International, Temecula, CA, USA) were used for indirect immunofluorescence, as previously described (50).

FJ (Histo Chem, Jefferson, AR, USA) has been shown to stain dying neurons in the brain (30,48) as well as other dying cells (31). Briefly, 10 μm sections were treated with 0.06% potassium permanganate in H₂O for 15 min and washed twice in H₂O and then immersed in FJ (0.001% FJ/0.1% acetic acid) for 30 min.

For assessment of cell replication in islets, animals received two daily injections of 5-BrdU (50 mg/kg) for 3 days and were perfused with 4% paraformaldehyde 1 day after the last injection. Occurrence of dividing cells was determined by immunostaining with an antibody recognizing 5-BrdU (1:20, Dako, Copenhagen, Denmark) in 10 μm cryostat sections. The total number of cells containing 5-BrdU-positive nuclei per islet was assessed (a total number of 300 islets/genotype, and five different animals per genotype were analyzed).

Islet number and β-cell volume were determined in an unbiased procedure, according to the principles of stereological morphometry. To this end, serial sections were prepared, and immunostained for insulin (9003), using the peroxidase–anti-peroxidase method as described (50). Islet numbers were determined by counting all islets in every 6th section of the whole pancreas. Total β-cell volume was determined in serial sections using Image ProMaster (measuring the area of 400 islets/genotype, three different animals per genotype). The investigator was unaware of the identity of the sections during analysis.

Electron microscopy

Small pieces of pancreatic tissue was fixed in a mixture of 1% glutaraldehyde and 3% paraformaldehyde in phosphate buffer, rinsed and postfixed for 1 h with 1% OsO₄, dehydrated in acetone and embedded in Polybed 812. Ultrathin sections
were cut and placed on copper grids before contrasted with 0.5% lead citrate and 4% uranyl acetate. Specimens were examined in a Philips CM10 transmission electron microscope.

**Electrophysiology**

The measurements were conducted using an EPC-10 patch-clamp amplifier in conjunction with the Pulse software suite (version 8.53; HEKA Elektronik, Lambrecht/Pfalz, Germany). The experiments were made using the standard whole-cell configuration of the patch-clamp technique, in which the cytosol is replaced by the pipette solution consisting of (in mM) 125 Cs-glutamate, 10 CsCl, 10 NaCl, 1 MgCl₂, 5 HEPES, 3 Mg-ATP, 0.1 cAMP and 0.05 EGTA (pH 7.2 with CsOH). Exocytosis was monitored as increases in cell capacitance (ΔC) in a control β-cell. Inset shows the voltage-dependent inactivation of the membrane Na⁺ current at membrane potentials < −70 mV, typical of β-cells. (B) ΔC was evoked by the respective depolarizations in the train stimulus. Data are mean ± SEM in 13 control β-cells. (C and D), as in (A and B), but data was obtained in 11 8-week-old R6/2 beta-cells. (E and F), as in (A and B), but data was obtained in seven 12-week-old R6/2 beta-cells.

Statistical analysis

Mean ± SEM are given. All data were analyzed using a two-tailed unpaired t-test or two factor analysis of variance (ANOVA) when appropriate. P < 0.05 was considered statistically significant.
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