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Expansion of the Golgi apparatus in rat cerebral cortex following intracerebroventricular injections of streptozotocin

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Abstract. Streptozotocin (STZ) is a bacterial toxin which selectively damages both insulin-producing cells and insulin receptors. Injections of STZ into the cerebral ventricles of experimental animals are followed by sustained biochemical, metabolic and behavioral effects resembling those which are found in human brains afflicted by Alzheimer's disease. The aim of the present study was to assess the effects of double intracerebroventricular application of STZ on the ultrastructure of rat frontoparietal cortical neurons. The most prominent change, seen 3 weeks after STZ injection, was a significant enlargement of the Golgi apparatus caused by expansion of the *trans*-Golgi segment of the cellular protein secretory pathway. Morphometric analysis revealed that the area of the *trans* part of the Golgi complex in neuronal cells was increased more than two-fold (median values: 312×10^3 nm^3 in 14 neurons from control animals, and 846×10^3 nm^3 in 19 neurons from STZ-treated animals, P=0.0012), whereas that of the *cis* part did not significantly change. The effects of STZ did not resemble Golgi atrophy and fragmentation described in neurons from disease-prone brain structures of patients with Alzheimer's disease, but were similar to that observed after intravenous application of a non-metabolizable glucose analog 2-deoxyglucose. Considering that proamyloidogenic processing of beta-amyloid precursor protein may occur preferentially in the *trans*-Golgi segment, the observed early response of neuronal ultrastructure to desensitization of insulin receptors may predispose cells to form beta-amyloid deposits.

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Key words: Alzheimer's disease, Golgi apparatus, streptozotocin

INTRODUCTION

Streptozotocin (2-deoxy-2-(3-methy1-3-nitrosoureido)--D-glucopyranose, STZ) is an antibiotic derived from the soil bacteria *Streptomyces achromogenes* (Lewis and Barbiers 1960). It had been developed as an anticancer agent (White 1963). However, since the discovery of its diabetogenic effects following systemic application (Rakieten et al. 1963) STZ is now used mainly to induce diabetes in experimental animals. When injected intravenously or subcutaneously, it induces diabetes resembling human type 1 (IDDM) or type II (NIDDM) disease, depending on protocol particulars (see for example Ito et al. 1999 and the references cited therein). This is because STZ is toxic both to pancreatic insulin-producing β-cells (Wilson and Leiter 1990) and to insulin receptors (Kadowaki et al. 1984, Meyerovitch et al. 1989), although in each case the mechanism of toxicity appears to be different. Toxicity toward β-cells most likely involves DNA alkylation and poly(ADP)ribosylation and can be selectively blocked by nicotinamide to create the "pure" animal model of NIDDM (Masiello et al. 1998), while toxicity toward insulin receptors is probably mediated by free radical mechanisms.

The brain responds poorly to systemic STZ application. For example, in STZ-diabetic rats the content of insulin receptors was increased in liver, kidney and skeletal muscle, but unchanged in neocortex (Pezzino et al. 1996). In a recent magnetic resonance spectroscopic study no changes in cerebral energy metabolism were observed after up to 8 months of STZ-induced diabetes, although some reductions in N-acetylaspartate levels in the brain were seen indicating metabolic or functional abnormalities in cerebral neurons (Biessels et al. 2001). However, STZ given intracerebroventricularly (i.c.v.) exerts profound and long-lasting influence on brain biochemistry, metabolism and function, including, *inter alia*, decreased glucose uptake and energy consumption, increased lactate output, decreased phospholipid content, tissue oxidative stress, cholinergic deafferentation, and compromised motor and cognitive performance (Duelli et al. 1994, Hoyer and Lannert 1999, Muller et al. 1998, Prickaerts et al. 1999, Sharma and Gupta 2001a). Collectively all these effects, which are long-lasting, resemble those seen in humans suffering from Alzheimer's disease (AD), and i.c.v. STZ has been *expressis verbis* proposed as a simple animal model of sporadic AD (Hoyer et al. 2000). The results of animal experiments with i.c.v. STZ have also been quoted as the evidence supporting the "glucose resistance" hypothesis of AD (Hoyer 2002a,b), according to which the dysfunction of brain insulin receptors is a primary pathogenetic event in AD (Hoyer 2002a,b, Meier-Ruge and Bertoni-Freddari 1996).

The "glucose resistance" hypothesis is in opposition to the most popular "amyloid cascade" hypothesis according to which a primary pathogenetic event in AD is formation of insoluble, neurotoxic amyloid beta-peptide (Abeta) deposits which results from imbalance between Abeta production and catabolism (Hardy and Higgins 1992, Hardy and Selkoe 2002). Abeta is derived from beta-amyloid precursor protein (β-APP) post-translationally modified and proteolytically cleaved within the endoplasmic reticulum and Golgi apparatus (Mattson 1997, Selkoe 2001). In Alzheimer's brains the Golgi apparatus of neurons from areas affected by the disease has been found fragmented and atrophic (Stieber et al. 1996). This, and the observation concerning linkage of a Golgi-related gene to a very aggressive form of AD (Sherrington et al. 1995) led to the hypothesis that disintegrated and atrophic Golgi complex may display a defect in β-APP processing which could result in elevated production of the amyloidogenic Abeta peptide (Dal Canto 1996).

If brain resistance to insulin is the primary pathogenetic factor in AD, it may be a cause of fragmentation and atrophy of the Golgi complex, and Golgi complex pathology could link impaired brain glucose metabolism to the stimulation of amyloidogenic processing of β-APP. If this hypothesis is correct, brain insulin receptor desensitization induced by i.c.v. STZ should also induce neuronal Golgi atrophy and fragmentation. We were unable to find any data concerning the effects of STZ on brain cells' Golgi morphology. Interestingly, however, STZ given systemically has been reported to have little effect on the liver cells Golgi appearance, except of that it significantly decreased the size of this organelle and seemed to suppress its secretory activity (Sarnecka-Keller et al. 1980, Kordowiak 1986). In the experiments reported herein we describe the effects of i.c.v. STZ on the ultrastructural morphology of the fronto-parietal neurons, with particular emphasis on the changes of the Golgi apparatus.

METHODS

The protocol of the study was approved by the First Ethical Committee of the City of Warsaw. The present study was performed on outbred male Wistar rats,

230-350 g body weight, bred in our facility and kept in an air-conditioned room in 12/12 h light/dark cycle with free access to water and food *ad libitum*. For intracerebroventricular injections and brain tissue sampling the animals were anesthetized with the mixture of ketamine (70 mg/kg) and xylazine (20 mg/ml).

Streptozotocin (STZ) purchased from Sigma was dissolved directly before use in artificial cerebrospinal fluid to a concentration of 25 mg/ml. Rats were placed in a stereotaxic frame and after incising skin and muscles holes were drilled bilaterally in the skull over the lateral ventricles. STZ (0.5 mg per each side) or vehicle was injected to the vicinity of each lateral ventricle (0.8 mm caudally from bregma, 1.5 mm laterally from sutura sagittalis, 3.6 mm below the brain surface) with the use of a Hamilton micro-syringe. The volume injected was 20 µl/ventricle. Holes in the skull were secured with a bone wax and the skin was sutured. The stereotaxically-guided injections were repeated after 3 days. To avoid infections the rats were prophylactically treated with ceftriaxon (0.2 g per day).

Three weeks after the first injection of STZ or vehicle the animals were perfused *via* the left heart ventricle with a fixative consisting of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 at 20°C. Tissue for ultrastructural studies was sampled from the fronto-parietal lobe of cerebral cortex, approx. 5 mm rostrally from the injection site (Fig. 1). This area was chosen because, according to Duelli and coauthors (1994), in rat brain following i.c.v. STZ injection, glucose consumption is most severely affected in frontal and in parietal cortex. The tissues were fixed for 20 h, postfixed in a mixture of 1% OsO_4 and 0.8% $K_4Fe(CN)_6$, and processed for transmission electron microscopy. After dehydration in series of ethanol and propylene oxide, tissue specimens were embedded in Spurr resin. Ultrathin (50 nm) sections were examined with a JEM 1200EX electron microscope.

To quantify the effect of treatment on the Golgi complex, morphometric analysis was performed blindly on brain cortical tissue samples from 3 control and 3 STZ-treated rats; for comparison, brain cortical

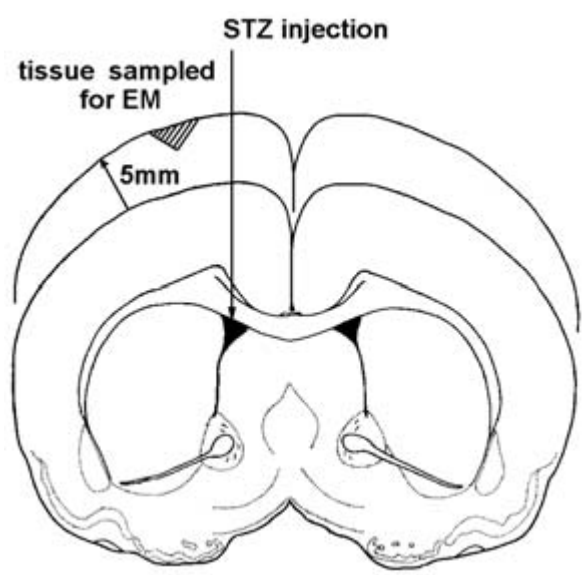

Fig. 1. A scheme showing the distance between the injection site and the site of sampling brain cortex for electron microscopy

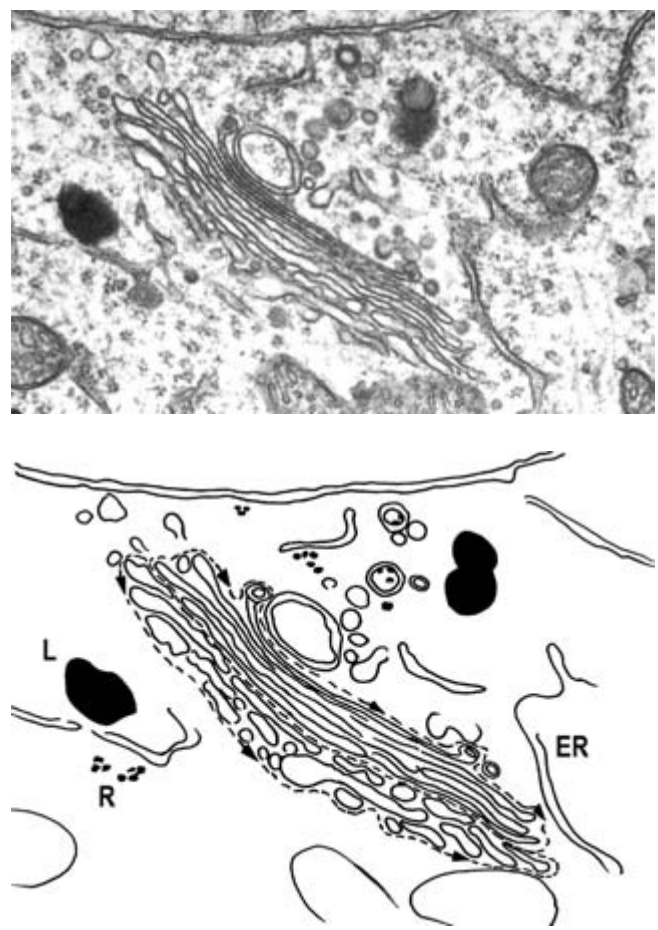

Fig. 2. Delineation of the *cis* and the *trans* Golgi. Upper panel: a micrograph of the Golgi in a neuronal cell at the high magnification. Lower panel: a sketch of the contours of organellae seen in the micrograph above. Broken line with arrows is the outline of the *cis* and the *trans* part of the Golgi, which consist of the long and parallel cisterns and of less regular and shorter tubules and vesicles described in literature as the saccotubular network, respectively. (L) lysosome; (ER) endoplasmic reticulum; (R) ribosomes.

tissue from 3 rats treated with 2-deoxyglucose as described in the preceding paper (Grieb et al. 2004) have also been analyzed. Tissue blocks with regions of well-preserved Golgi were returned to the microtome and ribbons of 5-6 serial 50 nm sections were cut. Each of these sections were then micrographed at high magnification (50 000 ×). A Microscan software package for the analysis of microscopic pictures (Centrum Mikroskopii, Warsaw, Poland) was used for determination of the size of the Golgi system in the neurons. On each micrograph the contours of the *cis* (i.e., proximal) and *trans* (i.e., distal) part of the Golgi complex were outlined as shown on Fig. 2, and their respective areas (expressed in square nanometers) were determined. The data for each series of sections were averaged and analyzed further as a single result concerning a given neuron. The Golgi complexes in 14 neurons from control animals, in 19 neurons from STZ-treated and in 25 neurons from 2-DG-treated animals were measured. Statistical analysis of the morphometric data was performed using Statistica 6 (Statsoft) software package, taking $P<0.05$ as the indication of significance.

RESULTS

In the fronto-parietal cortex samples taken from control rats a normally appearing brain tissue was found including neurons with visible Golgi apparatuses in which

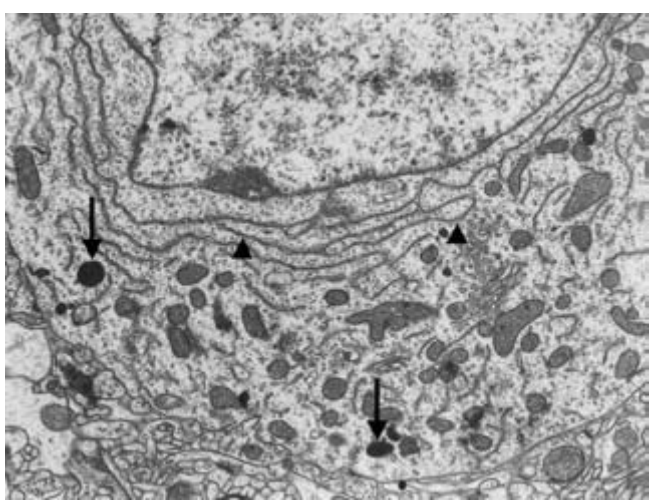

Fig. 3. Three weeks after the first i.c.v. injection of STZ. The perikaryal parts of the neuron. A single long endoplasmic reticulum (arrow heads) is seen in the vicinity of the nucleus. In cytoplasm aggregates of ribosomes, and inclusions resembling deposits of lipofuscin are present (arrows). Magnification, × 12 000.

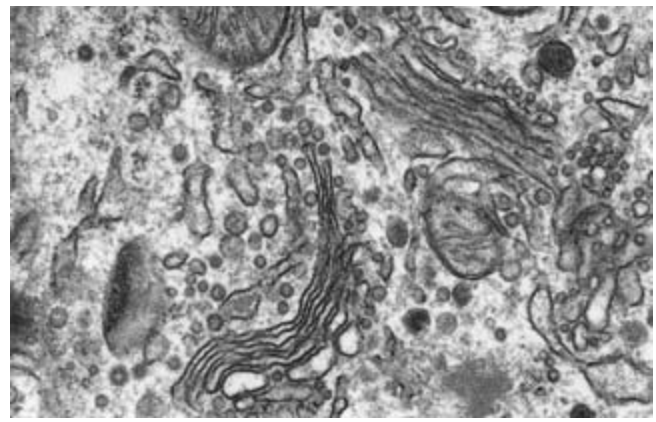

Fig. 4. Three weeks after the first i.c.v. injection of STZ. The part of the neuronal cell with an expanded Golgi system. An irregular dilatation of cisternae and aggregation of smooth microvesicles in the vicinity of the Golgi complex are seen. Magnification, × 50 000.

the *cis* and *trans* parts were most frequently of similar size. In the fronto-parietal cortex samples taken from rats injected intracerebroventricularly with streptozotocin characteristic morphological alterations in cellular organelle of the neurons prevailed. Endoplasmic reticulum was characterized by long, single cisternae present in the perinuclear zona (Fig. 3). The cisternae were devoid of ribosomes, which were dispersed in other parts of the cell. The mitochondria were not ultrastructurally changed. Cytoplasmatic inclusions resembling deposits of lipofuscin were frequently encountered (Fig. 4).

The most prominent ultrastructural feature of the cortical neurons from the cortex of i.c.v. STZ-treated rats

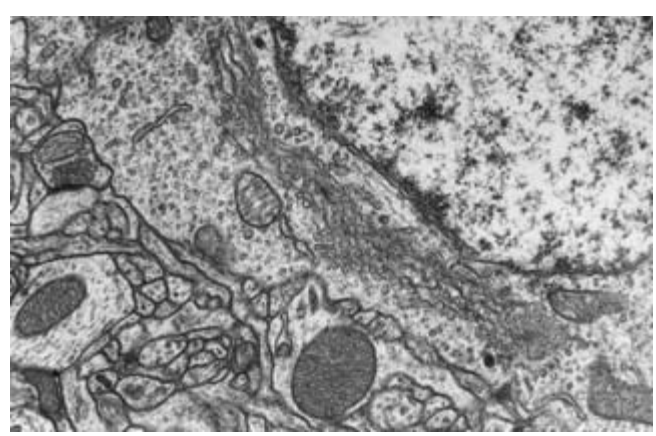

Fig. 5. Three weeks after the first i.c.v. injection of STZ. In the vicinity of nucleus the Golgi complex with characteristically expanded *trans*-Golgi network is present. Magnification, × 24 000.

was ultrastructural alterations of the Golgi apparatus. They were characterized by an increase in the number and size of cisternae and their irregular dilatation. In the *cis* face of the Golgi complex the single cisternae were dilated. Aggregates of smooth microvesicles were present in the *cis* and *trans* face. The *trans* part of the Golgi complex was visibly expanded, presenting itself as a large saccotubular network (Fig. 5). No signs of Golgi atrophy in cortical neurons were ever seen.

Results of the morphometric analysis are presented in Table I. Because in all but 4 cases the distribution of variables was significantly different from normal (Shapiro--Wilks $P<0.05$), the non-parametric Kruskal-Wallis ANOVA test was employed to evaluate significance of differences. With the exception of the area of the *cis* part of the Golgi complex which did not change due to treatments, all treatment effects were significant, indicating a prominent expansion of the *trans* (i.e., distal) part of this organellum. However, the effects of streptozotocin were much more pronounced than the effects of 2-deoxyglucose.

DISCUSSION

In our experiments the site of STZ injection was relatively distant from the site of cortical tissue sampling for EM (see Fig. 1). One may argue that the toxin injected into the cerebral lateral ventricles may act locally rather than diffuse across the whole bulk of the brain. Although such a possibility cannot be excluded, decomposition of STZ in biological milieu may involve generation of nitric oxide (Kwon et al. 1994), methyldiazohydroxide and alkylating methyldiazonium and carbonium cations (Wilson and Leiter 1990). It seems conceivable that these highly diffusible metabolites easily penetrate across brain extracellular spaces and reach locations distant from the injection site.

Metabolic and behavioral disturbances which follow single or double i.c.v. STZ injection(s) are long-lasting.

Table I

The size of the Golgi apparatus and its subcompartments in cerebral cortical neurons

Treatment (number of neurons evaluated)	Control (n=14)	2-DG (n=25)	STZ (n=19)
Whole Golgi ($\times 10^3$ nm^2)			
Mean (SD)	832 (609)	961 (378)	1 402 (813)
Median (lower quart. upper quart.)	580 (401; 1 287)	907 (691; 1 211)	1 249 (777; 1 556)
Normal distribution	No ($P<0.02$)*	Yes	No ($P<0.005$)
		Treatment effect: P=0.0174**	
Cis part of Golgi ($\times 10^3$ nm^2)			
Mean (SD)	366 (262)	317 (122)	439 (303)
Median (lower quart.; upper quart.)	258 (206; 455)	284 (205; 405)	369 (227; 538)
Normal distribution	No ($P<0.02$)	Yes	No ($P<0.006$)
		Treatment effect: ns	
Trans part of Golgi ($\times 10^3$ nm^2)			
Mean (SD)	467 (382)	645 (304)	962 (529)
Median (lower quart.; upper quart.)	312 (193; 716)	524 (481; 799)	846 (565; 1 018)
Normal distribution	No ($P<0.02$)	No ($P<0.003$)	No ($P<0.006$)
		Treatment effect: P=0.0012	
Trans/Cis ratio of areas			
Mean (SD)	1.27 (0.44)	2.17 (0.92)	2.50 (0.89)
Median (lower quart.; upper quart.)	1.25 (0.93; 1.53)	1.96 (1.58; 2.55)	2.13 (1.89; 3.11)
Normal distribution	Yes	No ($P<0.003$)	Yes
		Treatment effect: P=0.0001	

*Shapiro-Wilk's test; **Kruskal-Wallis ANOVA

This can be explained by the inability of brain tissues to fully compensate for the damage inflicted to the insulin receptors by the toxin or its metabolite(s). However, the presence of tissue oxidative stress in brain 3 weeks after i.c.v. STZ (Sharma and Gupta 2001a) and the ability of various antioxidants to ameliorate the effects of i.c.v. STZ (Sharma and Gupta 2001b, 2002, Veerendra Kumar and Gupta 2003) cannot be directly related to the injection of the toxin which is supposed to act transiently and extracellularly. In diabetes mellitus tissue oxidative stress has been attributed to hyperglycemia, but this explanation does not apply to the case of STZ injected into the cerebral ventricles, because systemic glycemia is not affected. Sustained oxidative stress in brain tissues (Sharma and Gupta, 2001a) and cytoplasmatic lipofuscin-like deposits which we have found following i.c.v. injections of STZ (free radical-mediated mechanisms are implicated in the generation of lipofuscin (Brunk and Terman 2002)) seem to reflect changes in brain metabolism which somehow result in excessive generation of free radicals.

When neurons degenerate, it seems rather obvious that their metabolic activity, including activity of the Golgi apparatus, would be diminished. Therefore, the atrophy and fragmentation of this organelle seen in neurons from human AD-afflicted brains (Stieber et al. 1996) may reflect the end-stage of the disease. In a series of papers (Salehi et al. 1994, 1995a,b,c, 1998) summarized in a review article of Salehi and Swaab (1999) extensive data concerning neurons of young and old AD patients as well as young and old controls were presented. These authors found no relationship between the size of the Golgi complex and age or severity of the cytoskeletal alterations in the neurons from nucleus tuberalis. However, in nucleus basalis of Meynert as well as in tuberomammilary neurons and in the CA1 sector of hippocampus the size of Golgi apparatus in AD patients was smaller than in controls. These data suggest that in AD there is reduced protein processing in neurons located in disease-affected areas, although it does not directly correlate with neuropathologic hallmarks of the disease such as neurofibrillary tangles. However, in vasopressin and oxytocin-producing magnocellular neurons of the supraoptic and paraventricular nucleus, which are not affected by the Alzheimer's disease, a significant increase in the size of the Golgi complex with age has been found (Lucassen et al. 1994) indicating that in the aging and failing brain neurons may still have some capacity to increase their protein processing capability.

We found no atrophy or fragmentation of Golgi apparatus in brain neurons of the rats injected i.c.v. with STZ. Instead, the most prominent ultrastructural feature of these cells was the apparent expansion of the *trans* part of the Golgi complex. A similar phenomenon has also been seen in cortical neurons following treatment with 2-deoxyglucose. Thus, in respect to Golgi complex morphology (at least at 3 weeks following injection of the toxin), the i.c.v. STZ model does not resemble Alzheimer's disease. It is, however, possible that the expansion of the *trans* part of the Golgi is a transient phenomenon which occurs only in pre-symptomatic AD, transforming later in the course of this disease into the fragmentation and atrophy of this organelle. This possibility requires further study.

The impression of a preferential expansion of the *trans* part of the Golgi complex has been confirmed by quantitative analysis, although our morphometric data must be interpreted cautiously. The Golgi complex is currently viewed as a series of four, or at least three subcompartments termed *cis*-, *medial*-, *trans*-Golgi, and *trans*-Golgi Network (TGN) (Glick 2000), or *cis*-Golgi network (CGN), stack of Golgi cisternae (STN) and TGN (del Valle et al. 1999). It remains unsettled whether these subcompartments are stable or formed through a continuous dynamic outgrowth and maturation of the Golgi cisternae, although recent evidence seems to favor the second model (Glick 2000). In either case the Golgi complex consists of a series of subcompartments which spread in an orderly fashion between its *cis*-face neighboring endoplasmatic reticulum and its *trans*- face directed toward the outer plasma membrane and differ from each other in biochemical composition of the membranes and physicochemical milieu inside the cisterns. The subcompartments may be localized with dedicated cytochemical techniques such as, lectinocytochemistry (Pavelka and Ellinger 1991), but in our morphometric analysis we did not use any such method. Instead, each Golgi area was divided into *cis* and *trans* parts on the basis of the gross structural criteria only. One may argue that such an approach provides data of a limited value. Interestingly, however, in experiments with cultured NRK (normal rat kidney) cells depletion of the cellular ATP pool to 15-25% of the control has been reported to result in a specific disassembly of the CGN (i.e., the part neighboring endoplasmic reticulum), whereas the other parts of the Golgi appeared energy-depletion insensitive (del Valle et al. 1999). When the same cell system was exposed to

20°C to block the transport of proteins out of the Golgi complex, the dominant feature of Golgi structure were the changes in the *trans*-most cisternae (Ladinsky et al. 2002). These data show that changes in gross Golgi morphology can be stimulus-specific and, depending on the stimulus, either the *cis* or *trans* part of the Golgi complex can be preferentially affected. Our morphometric evaluation (which was performed blindly) unequivocally supports the impression that treatment with both i.c.v. STZ and (to a lesser extent) i.v. 2-deoxyglucose produce preferential enlargement of the *trans* part of the Golgi complex. A common mechanism may be employed by which STZ as well, as 2-DG influence Golgi morphology. This issue is discussed in the accompanying paper (Grieb et al. 2004).

A possibility that the relative enlargement of the *trans* part of the Golgi complex may influence proteolytic processing of β-APP may also be considered. This process is compartmentalized along the secretory pathway of neurons so that distinct populations of Abeta amyloid peptides are generated in the endoplasmic reticulum and in the Golgi complex (Greenfield et al. 1999). It has recently been suggested that a very highly amyloidogenic truncated amyloid species Abeta(11-40/42), which accumulates in Alzheimer's brains, is preferentially generated in the *trans*-Golgi complex (Huse et al. 2002). The expansion of the distal part of the neuronal secretory pathway in response to brain insulin receptors' desensitization may provide for a relatively longer residence of β-APP in the *trans*-Golgi environment. This may result in increased generation of the truncated, particularly amyloidogenic form of Abeta. Such a mechanism could provide a link between the development of brain insulin receptor desensitization and the stimulation of amyloidogenesis during the early phase of the Alzheimer's disease.

CONCLUSIONS

Three weeks after double intracerebroventricular injection of streptozotocin in rats a significant expansion of the *trans*-Golgi part of the secretory pathway in cortical neurons was observed. This effect was similar, but more pronounced than the effect of intravenous 2-deoxyglucose seen 7.5 h after injection. Such a picture is very different from Golgi complex atrophy and fragmentation seen in Alzheimer's brains, but it may represent an early response to insulin receptor desensitization. Considering that a very highly amyloidogenic truncated amyloid species Abeta(11-40/42) is preferentially generated in the *trans*-Golgi complex, expansion of the distal part of the intracellular secretory pathway may facilitate amyloidogenesis.

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