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The long-acting GLP-1 derivative NN2211 ameliorates glycemia and increases β-cell mass in diabetic mice

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Rolin, Bidda, Marianne O. Larsen, Carsten F. Gotfredsen, Carolyn F. Deacon, Richard D. Carr, Michael Wilken, and Lotte Bjerre Knudsen. The long-acting GLP-1 derivative NN2211 ameliorates glycemia and increases β-cell mass in diabetic mice. *Am J Physiol Endocrinol Metab* 283: E745–E752, 2002. First published June 4, 2002; 10.1152/ajpendo.00030.2002.—NN2211 is a long-acting, metabolically stable glucagon-like peptide-1 (GLP-1) derivative designed for once daily administration in humans. NN2211 dose dependently reduced the glycemic levels in *ob*/*ob* mice, with antihyperglycemic activity still evident 24 h postdose. Apart from an initial reduction in food intake, there were no significant differences between NN2211 and vehicle treatment, and body weight was not affected. Histological examination revealed that β-cell proliferation and mass were not increased significantly in *ob*/*ob* mice with NN2211, although there was a strong tendency for increased proliferation. In *db*/*db* mice, exendin-4 and NN2211 decreased blood glucose compared with vehicle, but NN2211 had a longer duration of action. Food intake was lowered only on *day 1* with both compounds, and body weight was unaffected. β-Cell proliferation rate and mass were significantly increased with NN2211, but with exendin-4, only the β-cell proliferation rate was significantly increased. In conclusion, NN2211 reduced blood glucose after acute and chronic treatment in *ob*/*ob* and *db*/*db* mice and was associated with increased β-cell mass and proliferation in *db*/*db* mice. NN2211 is currently in phase 2 clinical development.

incretin hormones; diabetes; animal models; glucagon-like peptide-1

GLUCAGON-LIKE PEPTIDE-1 (GLP-1), an incretin hormone secreted from the intestinal L cells (23, 16), is highly effective in lowering blood glucose in type 2 diabetic patients (12, 21, 22, 28). The antihyperglycemic effects of GLP-1 are multifactorial, involving the pancreas, gastrointestinal tract, and brain. Thus GLP-1 is a potent insulin secretagogue (24), stimulating insulin release in response to a meal (7, 26) while concomitantly inhibiting glucagon secretion (24). Moreover, because these effects of GLP-1 are glucose dependent (36), the risk of severe hypoglycemia is minimal. GLP-1 also decreases gastric emptying (37) and reduces appetite (10), and more recent studies have shown that the hormone stimulates β-cell proliferation (5, 39, 27) and inhibits apoptosis (2, 13). Taken together, this spectrum of effects gives GLP-1 a unique physiological/pharmacological profile that is highly attractive as a basis for the development of an antihyperglycemic agent for the management of type 2 diabetes. However, GLP-1 is a substrate for dipeptidyl peptidase IV (DPPIV) (20, 3) and is rapidly inactivated and cleared from plasma (4), giving the native hormone a pharmacokinetic profile that is not optimal for therapeutic use. NN2211 is a novel long-acting GLP-1 derivative obtained by acylation of the GLP-1 molecule (17). The mechanisms of protraction of NN2211 are several. When injected subcutaneously, the compound is slowly released from the injection site. Once it enters the bloodstream, NN2211 is extensively bound to albumin, which protects it from degradation by DPPIV while at the same time reducing renal clearance. These characteristics combine to give the compound a plasma half-life of 14 h in pigs (17) and 10–12 h in humans (1, 14, 15), meaning that NN2211 possesses pharmacokinetic properties that may be suitable for once daily administration (17).

The aim of the present studies was to investigate the pharmacodynamics of NN2211 after acute and chronic dosing to diabetic (*ob*/*ob* and *db*/*db*) mice. We measured the effect on glycemia, food intake, and body weight as well as β-cell mass and β-cell proliferation rates. A comparison to another long-acting GLP-1 analog, exendin-4 (39), was made in one of the studies.

RESEARCH DESIGN AND METHODS

Animals

All experiments were carried out with permits from the Animal Experiments Inspectorate, Ministry of Justice, Denmark. Female *ob*/*ob* mice (Umeå strain) were obtained from M&B (Ll. Skensved, Denmark). Animals were 9–11 wk of age and had been diabetic for ~4–6 wk at the time of the actual

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experiment, with a weight range of 33.2–49.2 g. Female *db/db* mice (C57BL/Ks strain) were obtained from M&B, were 10–11 wk of age at the time of the experiment, and had a weight range of 33.1–50.0 g. Animals were housed (5–6 mice/cage in *studies 1* and *2* and 2 mice/cage in *study 3*) under controlled ambient conditions following a 12:12-h light-dark cycle, with lights on at 6:00 AM, and fed a standard Altromin no. 1324 diet (Brogaarden, Gentofte, Denmark) with free access to water. Animal health was subject to veterinary control. The animals were allowed 2 wk of acclimatization before initiation of the preexperiments, which accustomed them to the experimental procedures.

Preexperimental Period

To minimize stress due to handling, all animals were accustomed to blood sampling and dosing procedures for 1 wk before the start of the experiments.

Experimental Procedures

Study 1: dose response of NN2211 in ob/ob mice. Five groups of animals (n = 10–11) received a single subcutaneous injection (300 μl/50 g body wt) of either vehicle (0.9% NaCl solution containing 0.2% human serum albumin, pH 7.1) or NN2211 (30, 100, 300, or 1,000 μg/kg). NN2211 batch no. P971119A-9, purity 96.3%, was used, with concentrations being adjusted for purity of the compound.

Study 2: effect of chronic dosing with NN2211 in ob/ob mice. Two groups of animals (n = 10) received subcutaneous injections (300 μl/50 g body wt) of either vehicle (phosphate-buffered saline, pH 7.3–7.5) or NN2211 (100 μg/kg) twice daily (at 7:30 AM and 2:30 PM) for 2 wk (15 days). On the days of blood glucose monitoring, injections were given at 9:00 AM and 4:00 PM, respectively. NN2211 was dissolved in vehicle to give a concentration 16.7 μg/ml. NN2211 batch no. P971119A-9, purity 96.3%, was used, with concentrations being adjusted for purity of the compound.

Study 3: comparison of NN2211 and exendin-4 in db/db mice. Three groups of animals (n = 10) received subcutaneous injections (300 μl/50 g body wt) of either vehicle (phosphate-buffered saline, pH 7.3–7.5), NN2211 (200 μg/kg, batch no. NN221119801), or exendin-4 (100 μg/kg, batch no. H8730-515049, purity 83%; Bachem, Bubendorf, Switzerland) twice daily (at 7:30 AM and 2:30 PM) for 2 wk (15 days). On the days of blood glucose monitoring, injections were given at 9:00 AM and 4:00 PM, respectively. NN2211 and exendin-4 were dissolved in vehicle to give concentrations of 33.3 μg/ml and 16.7 μg/ml, respectively.

Analytical Procedures

Glucose. Blood glucose (BG) concentrations were measured in a 5-μl blood sample taken from the tip of the tail. The blood was collected into a heparinized capillary tube, shaken into a glucose buffer solution, and analyzed in an autoanalyzer (EBIO 6666; Radiometer, Copenhagen, Denmark) by a glucose oxidase method. BG was measured at the following time points: at 0, 2, 4, 6, 8, 10, and 24 h after acute dosing in *study 1*; at the same time points in *study 2* on *days 1*, *8*, and *15*; and at 0, 2, 4, 6, 8, 10, 12, 14, and 24 h after dosing on *days 1*, *8*, and *15* in *study 3*.

Insulin. A blood sample was obtained at the termination of the study, after decapitation during CO_2 anesthesia, and collected in heparinized chilled tubes containing 35 μg·ml aprotinin^{-1}·1 ml blood^{-1} (*study 2* only). Plasma insulin concentrations were measured with an in-house ELISA method by use of guinea pig antibodies GP114 and GP116 as primary and secondary antibodies and purified rat insulin (Novo Nordisk batch no. 220891) as standard. The detection limit of the assay was 3 pM. Both insulin type 1 and type 2 were measured equally. The inter- and intra-assay variations were 6.4 and 6.2%, respectively, at 1,650 pmol/l; 5.4 and 8.4%, respectively, at 330 pmol/l; and 2.0 and 10%, respectively, at 55 pmol/l.

Food and water. In *studies 1* and *3*, food intake and water intake (*study 3* only) were measured at 9:00 AM during the preexperimental period (8 days) and during the whole experimental period. In *study 2*, food intake was measured every 24 h during the experimental period only.

Body weight. Body weight was measured before dosing and 24 h after dosing in *study 1* and on *days 1*, *8*, and *15* in the 2-wk study period (*studies 2* and *3*).

Histology. Four hours before they were killed, the mice were injected intraperitoneally with 100 mg/kg of bromodeoxyuridine (BrdU; Sigma, St. Louis, MO). At death, the pancreas was taken out en bloc with the intestines and fixed in 4% paraformaldehyde for 24 h and then dissected free of surrounding tissue, weighed, and embedded in paraffin. Sections (3 μm) were deparaffinized and rehydrated, and endogenous peroxidase was blocked by H_2O_2 in ethanol, after which sections were treated with avidin and biotin. After microwave oven treatment in citrate buffer (pH 6) for 3 × 5 min at 90°C, sections were stained in an Autostainer (DAKO, Glostrup, Denmark) for BrdU and insulin with the use of mouse anti-BrdU, biotinylated goat anti-mouse Ig, and Vectastain streptavidin peroxidase (Vector) and were developed with diaminobenzidine (DAB) and $NiSO_4$. This was followed by treatment with guinea pig anti-insulin (ICN) and peroxidase-coupled rabbit anti-guinea pig Ig and development with Nova Red (Vector). For non-β-cells, sections were stained with the combination of mouse monoclonal antiglucagon, rabbit antisomatostatin, and rabbit antipancreatic polypeptide, followed by the combination of biotinylated swine anti-rabbit IgG, goat anti-mouse IgG, and streptavidin peroxidase and development with DAB and $NiSO_4$. All reagents, including normal sera for blocking, were from DAKO (Copenhagen, Denmark), if not mentioned otherwise. Stereological estimations were carried out on two sections cut 250 μm apart at an on-screen magnification of ×960. Sections were scanned in a random systematic way by use of CastGrid V2.0 (Olympus, Copenhagen, Denmark) to control the stage and the data collection. β-Cell BrdU index was estimated by analysis of 2,000 β-cells in the *ob/ob* pancreata and ~1,500 β-cells in the *db/db* pancreata for the presence of the BrdU staining. β- and non-β-cell volumes were estimated by point counting by using a grid system with 1 × 144 points. The pancreas area/volume was estimated via the 1-point grid/frame, and 400–700 hits were obtained on the two sections combined. The β-cell area/volume was estimated with the 144-point grid, and 600–3,500 hits were obtained from the two sections combined. The mass of the total pancreas of β- and non-β-cells was calculated by taking the grid ratio into consideration. The total mass of β- and non-β-cells (in mg) was calculated by multiplication by the pancreas weight.

Statistical analysis. Statistical analyses were made using the Student's t-test or the nonparametric Kruskal-Wallis test, followed by the Mann-Whitney test to compare two groups/data sets where significant differences were found. For comparisons of more than two groups, a one-way ANOVA followed by a Tukey's multiple comparisons test was made. P value < 0.05 was taken to represent statistical significance. Data are expressed as means ± SE. Calculations of area under the curve (AUC) were made using baseline = 0.

RESULTS

Study 1

BG. Figure 1 shows BG concentrations after administration of either vehicle or NN2211 at different doses. In the NN2211-treated groups, there were dose-dependent decreases in BG during the experiment, with all groups having significantly lower AUC for BG: 30 μg/kg, 361 ± 39 mmol·l^{-1}·h ($P < 0.001$); 100 μg/kg, 348 ± 45 mmol·l^{-1}·h ($P < 0.001$); 300 μg/kg, 279 ± 30 mmol·l^{-1}·h ($P < 0.0001$)l; and 1,000 μg/kg, 283 ± 27 mmol·l^{-1}·h ($P < 0.0001$) compared with vehicle (524 ± 33 mmol·l^{-1}·h). At 24 h postdose, BG was still significantly ($P < 0.01$) reduced compared with the vehicle group, but a complete normalization of BG did not occur with any dose.

Food intake. Figure 2 shows the average 24-h food intake per animal (5–6 mice/cage) during the eight preexperimental periods (baseline) compared with the food intake on the day of the experiment. Average baseline food intake varied from 41 to 53 g/cage during 24 h and was not different among the five groups. Vehicle treatment had no significant effect, but in contrast, administration of NN2211 caused a marked and dose-dependent decrease in food intake at all dose levels ($P < 0.05$), corresponding to reductions of 39 ± 1, 62 ± 7, 66 ± 4, and 66 ± 4% at doses of 30, 100, 300, and 1,000 μg/kg NN2211, respectively.

Body weight. Body weight was measured before and 24 h after administration of either vehicle or NN2211 and expressed as the body weight change (in g) per group over the 24-h postdosing period relative to the predosing body weight. A significant ($P < 0.001$) and dose-dependent decrease in body weight occurred in the NN2211-treated groups (by 1.0 ± 0.2, 1.7 ± 0.2, 2.5 ± 0.2, and 2.5 ± 0.2 g after doses of 30, 100, 300, and 1,000 μg/kg NN2211, respectively), corresponding to a maximal weight loss of between 5 and 6%, whereas body weight in the vehicle-treated group was not significantly altered (−0.4 ± 0.3 g).

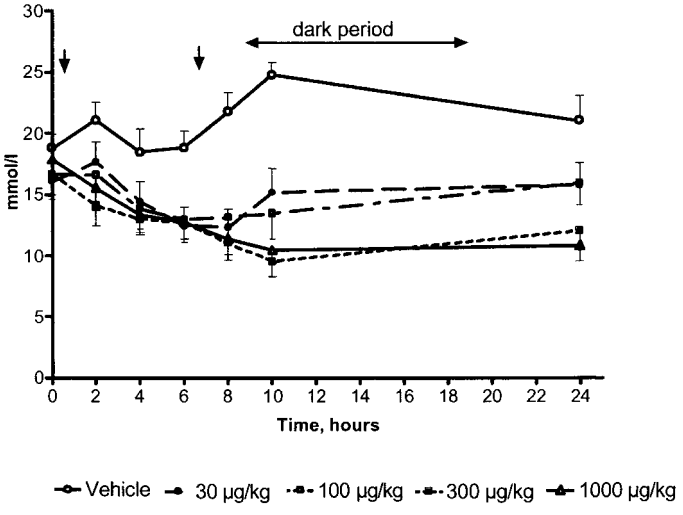

Fig. 1. Means ± SE for blood glucose (BG; in mmol/l) vs. time (in hours) after a single subcutaneous injection of 4 different dose levels of NN2211 or vehicle (n = 9–10). *Time 0* is at 9:00 AM. Arrows indicate injection times.

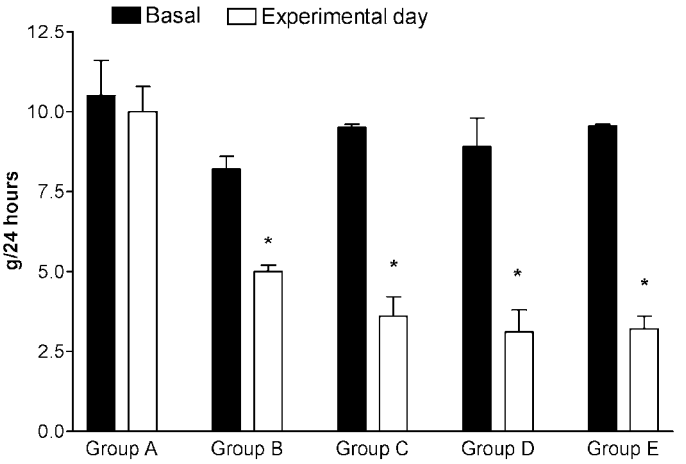

Fig. 2. Means ± SE for the average basal food intake per animal (in g/24 h) measured on 8 different preexperimental 24-h periods vs. the experimental 24-h period after administration of 4 different dose levels of NN2211 or vehicle (5–6 mice/cage; n = 2 cages/group). *Group A*, vehicle; *group B*, 30 μg/kg NN2211; *group C*, 100 μg/kg NN2211; *group D*, 300 μg/kg NN2211; *group E*, 1,000 μg/kg NN2211. *$P < 0.05$

Study 2

BG. The BG profiles after 1, 8, and 15 days of dosing with either vehicle or NN2211 (100 μg/kg twice daily) are shown in Fig. 3. When expressed as AUC for BG, significant reductions were obtained with NN2211 treatment compared with vehicle treatment on all days ($P < 0.001$). In both vehicle ($P < 0.0001$)- and NN2211 ($P < 0.0001$)-treated groups, glycemic levels increased over the 2-wk study period. However, compared with

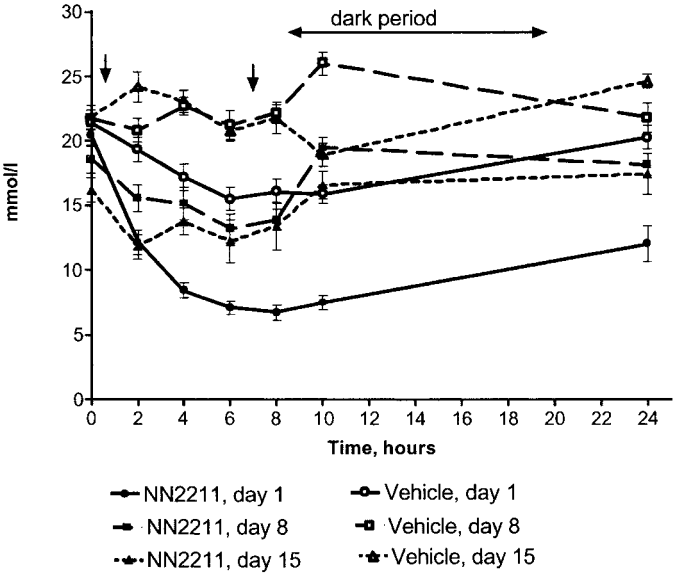

Fig. 3. Means ± SE for BG (in mmol/l) vs. time (in hours) during a 24-h period on *days 1*, *8*, and *15* of dosing of either NN2211 (100 μg/kg sc bid) or vehicle (n = 10). *Time 0* is at 9:00 AM. Arrows indicate injection times.

vehicle treatment, NN2211 significantly reduced the mean minimum and maximum BG concentration ($P < 0.001$) and the AUC for glucose throughout the study period, but a normalization of glycemic levels was not achieved.

Food intake. Apart from a tendency for food intake to be lower on the first day of dosing with NN2211, there were no significant differences between the two groups over the course of the study [AUC for food intake: 179 ± 15 g·24 h^{-1}·days (vehicle) vs. 133 ± 8 g·24 h^{-1}·days (NN2211) for the period of 0–4 days; and 636 ± 38 g·24 h^{-1}·days (vehicle) vs. 636 ± 14 g·24 h^{-1}·days (NN2211) for the period of 0–15 days].

Body weight. Body weight was not affected by either treatment and remained constant over the 15-day period (data not shown).

Plasma insulin. After 2 wk of dosing, plasma insulin was increased ($P < 0.01$) from 7,783 ± 862 pmol/l in the vehicle-treated group to 12,480 ± 1,180 pmol/l in the animals receiving NN2211, representing a 60% increase (measured 4 h after the last dose).

Histology. β-Cell proliferation rate as measured by BrdU staining was increased by NN2211 treatment in *ob/ob* mice, but the increase did not reach statistical significance ($P = 0.052$), whereas the β-cell mass was unaffected (Fig. 4). There was no difference between β-cell mass or relative β-cell mass, as the pancreas weights and the body weights did not differ between the groups (data not shown). There was no apparent difference in number of BrdU-positive duct cells and exocrine cells, nor was there any difference in the insulin staining intensity.

Study 3

BG. Figure 5 shows the BG profile after 1, 8, and 15 days of dosing of vehicle, NN2211 (200 μg/kg), or exendin-4 (100 μg/kg) twice daily, and the AUC are summarized in Table 1. All three groups showed a deterioration in glucose tolerance over the 2-wk period

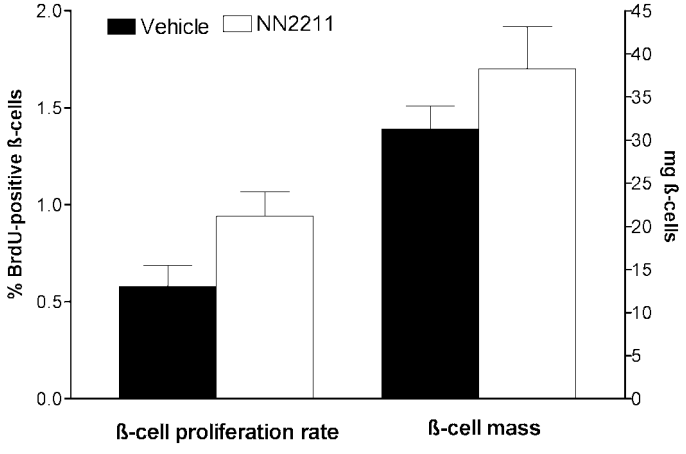

Fig. 4. *Left*: means ± SE for the percentage of bromodeoxyuridine (BrdU)-positive β-cells after 15 days of dosing with either NN2211 (100 μg/kg sc bid) or vehicle ($n = 10$). *Right*: means ± SE for the mass of β-cells expressed in mg/total pancreas after 15 days of dosing with either NN2211 (100 μg/kg sc bid) or vehicle.

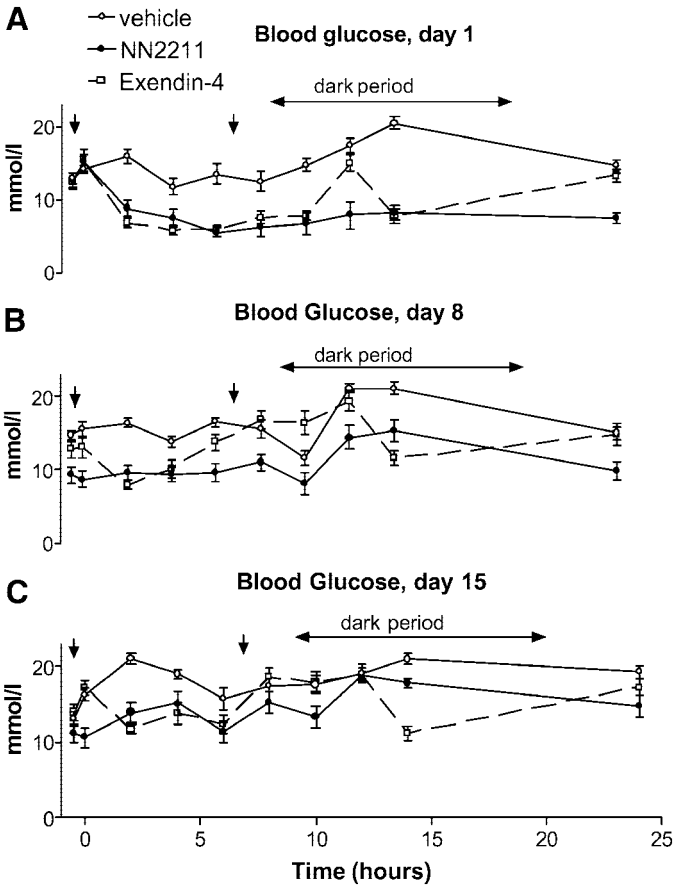

Fig. 5. Means ± SE for BG (in mmol/l) vs. time (in hours) during a 24-h period on *day 1* (*A*), *day 8* (*B*), and *day 15* (*C*) of dosing of NN2211 (200 μg/kg sc bid), exendin-4 (100 μg/kg sc bid), or vehicle ($n = 10$). *Time 0* is at 9:00 AM. Arrows indicate injection times.

($P < 0.01$). Compared with vehicle treatment, both exendin-4 and NN2211 significantly decreased the glucose AUC throughout the study period (although this failed to reach statistical significance for exendin-4 treatment on *day 8*). Both compounds exhibited a similar and maximal effect 6 h postdose, but in the exendin-4-treated group, BG levels returned to vehicle levels after 10–12 h, whereas NN2211 treatment maintained the BG at a significantly lower level compared with vehicle throughout the glucose monitoring period.

Food intake. Both NN2211 and exendin-4 reduced food intake significantly on *day 1* ($P < 0.01$ compared with vehicle), from 17.4 ± 1.5 g·$cage^{-1}$·24 h^{-1} in the vehicle-treated group to 6.4 ± 0.9 and 7.6 ± 0.8 g·$cage^{-1}$·24 h^{-1} in animals receiving exendin-4 or

Table 1. *Study 3: db/db mice*

Dosing	Vehicle	NN2211	Exendin-4
Day 1	389 ± 16	193 ± 24‡	233 ± 20*
Day 8	415 ± 11	278 ± 28†	332 ± 22
Day 15	467 ± 17	373 ± 24*	366 ± 18†

Values are means ± SE for 24-h areas under the curve for glucose (in mmol·l^{-1}·h). *$P < 0.01$, †$P < 0.001$, and ‡$P < 0.0001$ vs. vehicle.

NN2211, respectively, with two mice per cage. However, this effect was not maintained, so that during the second week of the study, there was no difference among the groups (average daily food intake during 7–14 days: 13.4 ± 0.9, 16.5 ± 0.5, and 17.0 ± 0.5 $g \cdot cage^{-1} \cdot 24\ h^{-1}$ in vehicle, exendin-4-, and NN2211-treated groups, respectively).

Water intake. Water intake was reduced significantly ($P < 0.05$) with NN2211 treatment during the second week of dosing (8.2 ± 0.7 $g \cdot cage^{-1} \cdot 24\ h^{-1}$) compared with vehicle treatment (14.3 ± 1.7 $g \cdot cage^{-1} \cdot 24\ h^{-1}$), but there was no significant effect of exendin-4 treatment (11.4 ± 1.3 $g \cdot cage^{-1} \cdot 24\ h^{-1}$). There were two mice per cage in all groups.

Body weight. Body weight showed a nonsignificant trend toward a reduction over the study period in exendin-4- and NN2211-treated animals compared with vehicle treatment (data not shown).

Histology. NN2211 treatment resulted in significantly ($P < 0.05$) increased β-cell mass and significantly ($P < 0.001$) increased β-cell proliferation rate as measured by BrdU incorporation compared with vehicle. Exendin-4 treatment resulted in a significant ($P < 0.05$) increase in the β-cell proliferation rate, but there was only a nonsignificant trend toward an increase in β-cell mass (Fig. 6).

DISCUSSION

In this study, we have shown that the long-acting GLP-1 derivative NN2211 has significant antihyperglycemic effects after both acute and chronic administration in two murine models of diabetes, namely the *ob*/*ob* and the *db*/*db* mouse. Moreover, the proportion of β-cells was increased after 2 wk of treatment with NN2211 in *db*/*db* mice but not with another GLP-1 analog, exendin-4, which most likely reflects the beneficial effects of the long duration of action offered by NN2211. However, exendin-4 has been reported to increase both β-cell proliferation rate and β-cell mass in other animal models (39, 35)

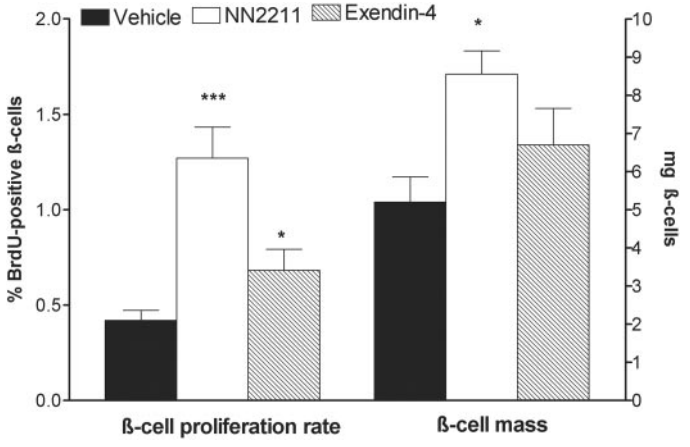

Fig. 6. *Left*: means ± SE for the percentage of BrdU-positive β-cells after 15 days of dosing with NN2211 (200 μg/kg sc bid), exendin-4 (100 μg/kg sc bid), or vehicle ($n = 10$). *Right*: means ± SE for the mass of β-cells expressed in mg/total pancreas after 15 days of dosing with NN2211 (200 μg/kg sc bid), exendin-4 (100 μg/kg sc bid), or vehicle ($n = 10$).

After acute treatment, NN2211 reduced BG in a dose-dependent manner, with a dose of 300 μg/kg having maximal effect, but a complete normalization of glycemic levels was not obtained. Likewise, on chronic treatment, BG levels were reduced but not normalized by NN2211. However, this finding was not unexpected, as the animals used in this study were severely diabetic and insulin resistant. Thus the present results obtained with NN2211 are in accordance with the findings of Greig et al. (11), who showed that chronic exendin-4 treatment did not fully normalize glycemic levels in *db*/*db* mice, even after 13 wk of treatment. It has been shown in vitro that GLP-1 and NN2211 are equipotent (17). However, NN2211 has reduced potency in vivo compared with native GLP-1 because of the extensive albumin binding of NN2211 in plasma, which is responsible for its protracted kinetic profile (17). Albumin binding thus acts as a reservoir from which the active drug can dissociate, resulting in a plasma half-life in the order of 4 h after subcutaneous administration in rats (unpublished data), with corresponding values of 14 h in pigs (17) and 10–12 h in humans (1, 15). The half-life of NN2211 after intravenous administration to humans is in the order of 8 h (14). This contrasts with the considerably shorter half-life of 26 min for exendin-4 (6) and only 1- to 1.5-min for active GLP-1 (4). Pharmacokinetic data after subcutaneous injection to mice are not published for NN2211 or exendin-4; however, assuming that the human situation is reflected in a mouse, the half-life of exendin-4 is expected to be considerably shorter than the half-life of NN2211. A narrow therapeutic window is expected when targeting the GLP-1 receptor because of dose-related side effects (nausea), which have been shown for exendin-4 (6). It is possible that protracted duration of action, avoiding peak concentrations of NN2211, will result in a reduction in the incidence of side effects. The prolonged plasma survival time of NN2211 explains its long duration of action, with BG levels still being significantly lower compared with vehicle treatment even 24 h after dosing. For practical reasons, the injections were given twice daily during the daytime, 7 h apart. Although this may not have been the most optimal dosing regimen seen in relation to rodent eating patterns, it seems justifiable, as the two mice strains used in these studies are strongly hyperphagic and thus have a disturbed eating pattern compared with normal rodents. It is our experience (unpublished data) that these mice eat considerable amounts of their daily food intake during the daytime. The antihyperglycemic effects of NN2211 were maintained, with BG being significantly lower throughout the dosing period in the NN2211 animals. However, it is noteworthy that there was a trend for the effect to become somewhat less pronounced than on *day 1*. It is likely that this is explained by the fact that the food intake lowering effect of both NN2211 and exendin-4 disappeared after 4 days. This is supported by a study in Zucker diabetic fatty (ZDF) rats in which pair feeding was used to

demonstrate that a reduction in food intake of equivalent magnitude to that produced by NN2211 accounted for ~50% of the reduction in BG (32). In the acute *study 1*, NN2211 reduced food intake in *ob*/*ob* mice in a dose-dependent manner. This effect was very marked, resulting in a weight loss of up to 6%. However, it is unlikely that over this short (24 h) period the reduced food intake was the only factor responsible for the reduction in body weight, and one might speculate that fluid loss also contributed. GLP-1 has been demonstrated to reduce water intake and stimulate urinary excretion of water and sodium acutely in normal rats (34), but it has been shown that with NN2211, these parameters are all normalized after 2 days of dosing (18). Although drinking behavior was not specifically addressed in a 13-wk study with exendin-4 in *db*/*db* mice, it was observed that the cages were drier after exendin-4 compared with vehicle treatment (11), perhaps suggesting that the diuretic effect does not persist. However, it could also reflect the net effect of reduced diuresis as a result of the concomitant reduction of glycemia caused by exendin-4 (11). Similarly, in the present study, the reduced water intake in *db*/*db* mice after 2 wk of treatment with NN2211 was associated with an improvement in their diabetes. Despite the initial reduction in food intake and body weight in the first 3–4 days of treatment, in the longer term these parameters were not significantly affected by NN2211 in either *ob*/*ob* or *db*/*db* mice. Other studies with exendin-4 have similarly noted a short-lived reduction in food intake and body weight in *db*/*db* mice, which, however, increased again to match vehicle treatment by *day 7* of treatment (11). In that study (11), it was suggested that exendin-4 may be causing taste aversion, rather than it being anorectic. This effect seems to be specific to mice, because in rats there appears to be no tachyphylaxis to the food intake and body weight effects of exendin-4 (40, 33) or NN2211 (32, 18, 30) over periods of up to 8 wk. Similarly, in a clinical study where native GLP-1 was infused continuously in obese type 2 diabetic subjects, a reduction in food intake and a significant weight loss were obtained after 6 wk of treatment (41). The exact mechanism by which NN2211 reduces food intake is unknown. GLP-1 is known to reduce gastric emptying (37), which in itself may limit food intake via neural or endocrine pathways associated with gastric distension or the presence of nutrients in the stomach (29). However, it has also been demonstrated that GLP-1 can reduce prospective food consumption and sensations of hunger and increase feelings of satiety even between meals (9, 10). Peripheral GLP-1 can access areas of the brain known to be associated with food intake (25), while other gastrointestinal effects of GLP-1 have been shown to be mediated via afferent vagal fibers to the brain (35), suggesting that GLP-1 may influence subjective appetite sensations either directly or by interacting with peripheral sensory nerve fibers. Plasma insulin was increased in *ob*/*ob* mice after 2 wk of treatment with NN2211, and similar findings were reported after 13 wk with exendin-4 in *db*/*db* mice (11), reflecting the insulinotropic action of these agents. However, one might have expected to find a reduction in basal plasma insulin levels as a consequence of the improvement in glycemic control. It is likely that the progressive nature of severe insulin resistance of these two murine models of diabetes can explain this finding, and it is noteworthy that 8 wk of exendin-4 administration in Zucker rats was associated with both reduced glycemia and reduced insulin levels, suggesting improved glucose tolerance (33). Similarly, a reduction in both glycemic levels and basal insulin concentrations was observed after 2 wk of treatment of 8-wk-old ZDF rats with NN2211 (30).

Histological examination of the pancreas showed a strong tendency toward an increased proliferation rate of β-cells in *ob*/*ob* mice after NN2211 treatment. In *db*/*db* mice, NN2211 treatment resulted in a significantly increased proliferation rate of β-cells and a significantly increased β-cell mass. GLP-1 has been shown to have positive direct effects on β-cell replication and neogenesis (5, 39, 27), and recent studies have also demonstrated that GLP-1, NN2211, and exendin-4 are able to inhibit β-cells apoptosis (2, 13). The net effect of GLP-1 or GLP-1 derivatives on β-cell proliferation and/or apoptosis in vivo is likely to be complex, since factors such as glycemia and lipidemia, which are themselves regulated by GLP-1, also affect β-cell growth and β-cell death (8, 19, 31). Therefore, the end result observed in vivo will be influenced by factors such as the age, the diabetic stage of the animals (whether there are many proliferating β-cells or many apoptotic-prone β-cells), the glycemic (and lipidemic) level obtained by treatment, and the duration of treatment. In *study 3*, NN2211 and exendin-4 both increased the β-cell mass (although the increase was only statistically significant for NN2211) and β-cell proliferation rate. The animals used in these studies were in a rather late stage of their diabetes and were still, despite treatment, hyperglycemic. Therefore, both the GLP-1 derivatives and the rather high glycemic level at this stage of the animals' lives will probably act in the same direction, namely to increase β-cell mass and β-cell proliferation rate. In the present study, a comparison between NN2211 and exendin-4 was made in *db*/*db* mice for the purpose of comparing the data with an earlier published study on exendin-4 in this model (11). We gave maximally effective doses of both compounds, which resulted in both GLP-1 derivatives lowering BG to a similar extent. The dose of NN2211 was chosen on the basis of the results obtained in *study 1* in *ob*/*ob* mice, and the dose of exendin-4 was chosen on the basis of data from the literature (11). The dose of NN2211 should be regarded in light of its large extent of albumin binding, with only 1–3% of the compound being free in plasma. NN2211 had a longer duration of action than exendin-4, a finding that can be explained by the different kinetic properties of the two compounds. Thus, although resistant to DPPIV, exendin-4 still has a relatively short plasma half-life of 26 min in humans (6) compared with the 8 h after intravenous administration for NN2211 (14). This is likely because

exendin-4, in contrast to NN2211, is not albumin bound and is, therefore, still subject to renal clearance. The finding that the metabolic clearance rate of exendin-4 is of similar magnitude to the glomerular filtration rate (6) further supports the suggestion that the metabolic stability of exendin-4, like native GLP-1 (21), is ultimately regulated by the kidneys. Therefore, because of the longer duration of action of NN2211, these animals probably obtained better glycemic control throughout the study period compared with animals treated with exendin-4. This is reflected in the significantly decreased water intake in NN2211 treatment (reflecting reduced urine output) and probably also the significantly increased β-cell proliferation rate and proportion of β-cells in the NN2211 animals.

In summary, the GLP-1 derivative NN2211 significantly reduced BG after both acute and chronic treatment in two murine models of diabetes. This was associated with an increase in β-cell mass and β-cell proliferation rate in *db*/*db* mice. NN2211 had a more protracted duration of action compared with another GLP-1 analog, exendin-4, reflecting the longer pharmacokinetic half-life of NN2211. These data, therefore, support the development of NN2211 as a new therapeutic agent suitable for once daily administration to patients with type 2 diabetes. NN2211 is currently in phase 2 clinical development.

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