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Metabolic and neuroprotective effects of dapagliflozin and liraglutide in diabetic mice

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Abstract

This study assessed the metabolic and neuroprotective actions of the sodium glucose co-transporter-2 inhibitor dapagliflozin in combination with the GLP-1 agonist liraglutide in dietary-induced diabetic mice. Mice administered low-dose streptozotocin (STZ) on a high fat diet received dapagliflozin, liraglutide, dapagliflozin-plus-liraglutide (DAPA-Lira) or vehicle once-daily over 28 days. Energy intake, body weight, glucose and insulin concentrations were measured at regular intervals. Glucose tolerance, insulin sensitivity, hormone and biochemical analysis, dual-energy x-ray absorptiometry densitometry, novel object recognition, islet and brain histology were examined. Once-daily administration of DAPA-Lira resulted in significant decreases in body weight, fat mass, glucose and insulin concentrations, despite no change in energy intake. Similar beneficial metabolic improvements were observed regarding glucose tolerance, insulin sensitivity, HOMA-IR, HOMA-β, HbA1c, and triglycerides. Plasma glucagon, GLP-1 and IL-6 levels were increased and corticosterone concentrations decreased. DAPA-Lira treatment decreased alpha cell area and increased insulin content compared to dapagliflozin monotherapy. Recognition memory was significantly improved in all treatment groups. Brain histology demonstrated increased staining for doublecortin (number of immature neurons) in dentate gyrus and synaptophysin (synaptic density) in stratum oriens and stratum pyramidale. These data demonstrate that combination therapy of dapagliflozin and liraglutide exerts beneficial metabolic and neuroprotective effects in diet-induced diabetic mice. Our results highlight important personalised approach in utilising liraglutide in combination with dapagliflozin, instead of either agent alone, for further clinical evaluation in treatment of diabetes and associated neurodegenerative disorders.

Keywords: dapagliflozin; diabetes; GLP-1; glucagon; liraglutide

Introduction

Type 2 diabetes mellitus (T2DM) is a metabolic disorder that arises due to a complex array of molecular defects manifesting in dysregulated insulin secretion, impaired insulin action, or both. Since the pathophysiology of T2DM is multifaceted and involves a range of biochemical mechanisms, there is no single therapy can effectively manage all aspects of the disorder (Zaccardi *et al*. 2016). Moreover, as T2DM and obesity levels are increasing at an alarming rate, more effective therapies and innovative treatment strategies are urgently needed to control glycaemia, reduce body weight and decrease the risk of micro- and macrovascular complications (da Rocha Fernandes *et al*. 2016). The previous two decades have witnessed a surge in the number of new drug classes such as glucagon-like peptide-1 (GLP-1) agonists, dipeptidylpeptidase-4 (DPP4) inhibitors and sodium-glucose-cotransporter-2 (SGLT2) inhibitors (Bailey *et al*. 2016). Although these agents may be used as monotherapies, it is becoming increasingly apparent that successful and cost effective management of T2DM requires development of safe combination therapies with distinct and complementary mechanisms of action.

The kidneys play a pivotal role in regulating glucose homeostasis as most of the glucose filtered by the glomerulus is reabsorbed (Gerich *et al*. 2001). In healthy subjects, the high capacity, low-affinity SGLT2, reabsorbs approximately 90% of glucose in S1 segment of proximal tubules (Hediger & Rhoads 1994, Han *et al*. 2008). Under conditions of chronic hyperglycaemic, SGLT2 is up regulated and this enhances glucose reabsorption and worsens glycaemia (Rahmoune *et al*. 2005). Dapagliflozin, a highly selective and potent oral inhibitor of SGLT2, reduces reabsorption of filtered glucose leading to increased glucosuria and improvement in glycaemic control (Vivian 2015). Although actions of dapagliflozin appear to be independent of insulin secretion, dapagliflozin improves insulin sensitivity, most likely as a result of sustained reduction in hyperglycaemia, alleviation of glucose toxicity and weight reduction through enhanced caloric loss (Macdonald *et al*.

2010, Mudaliar *et al*. 2014, Merovci *et al*. 2015, Millar *et al*. 2016). Beneficial actions of dapagliflozin are, to some extent, limited by unrestrained hepatic glucose production (Bonner *et al*. 2015). Thus, inhibition of hepatic glucose output by stimulation of insulin secretion as well as inhibition of glucagon secretion may significantly enhance therapeutic efficacy of SGLT2 inhibition.

GLP-1 agonists are well established as effective agents to treat patients with T2DM due to a range of beneficial actions including weight loss, induction of satiety, inhibition of gastric emptying, stimulation of insulin secretion and inhibition of alpha cell function (Bailey *et al*. 2016). In addition, GLP-1 agonists exert effects at other extra pancreatic sites (Renner *et al*. 2016), with notable neuroprotective actions in animal models of diabetes-obesity, Alzheimer's disease (AD) and Parkinson's disease (PD) (Ashraghi *et al*. 2016, Tramutola *et al*. 2017). Liraglutide (Victoza®) is a highly effective long-acting GLP-1 agonist that shares 97% sequence homology with human GLP-1 (Knudsen *et al*. 2000). Structural modifications include amino acid substitution of Lys^{34} with Arg, and addition of lipophilic C_{16} acyl moiety at position 26 via gamma-glutamyl linker (Madsen *et al*. 2007). These structural changes provide liraglutide with enhanced pharmacokinetic profile and significantly prolonged half-life, thus facilitating once-daily injection (Agersø *et al*. 2002). This prolonged bioactivity has been attributed to non-covalent reversible albumin binding, ability of liraglutide to self-aggregate and form heptamers in solution, and stability to the enzyme DPP4 (Knudsen *et al*. 2000, Madsen *et al*. 2007, Li *et al*. 2016).

Given the need for more personalised treatment strategies for patients with T2DM and the unique mechanism of action of dapagliflozin and liraglutide, we hypothesised that combining both drugs would provide additive metabolic and neuroprotective outcomes. We chose to administer a GLP-1 agonist rather than DPP-4 inhibitor as DPP-4 inhibitors act to prevent degradation of a number of regulatory peptides including GLP-1 (Bailey *et al*. 2016). As such, HF mice on

background low-dose STZ were treated with dapagliflozin or liraglutide as monotherapy and combination therapy for 28 days. Effects on glucose tolerance, insulin sensitivity, body weight, hormones, memory and learning, islet and brain histology were assessed.

Materials and methods

Animals

Male NIH Swiss mice (aged 8-10 weeks) purchased from Harlan (Oxon, UK) were kept at 22±2ºC with 12:12 h light/dark cycle. Mice had free access to high fat diet (45% AFE Fat; Product Code 824053; Special Diet Services, Witham, UK; total energy 26.15 kJ/g). An additional group of mice had free access to standard rodent chow (Teklad Global 18% Protein Rodent Diet; Product Code 2018S; Harlan, UK; total energy 13.0 kJ/g). All animals had free access to drinking water and no adverse effects were observed during the entire experimental study. All experiments were performed according to the *Principles of Laboratory Animal Care* (NIH publication no. 86-23, revised 1985) and UK Home Office Regulations (UK Animals Scientific Procedures Act 1986).

Experimental design

Mice commenced high fat diet on day -28 and subsequently received STZ (Sigma-Aldrich, Dorset, UK) prepared in sodium citrate buffer (pH 4.5) on day -14 (50 mg/kg; i.p.) and day -7 (50 mg/kg; i.p.). Mice that displayed a blood glucose concentration greater than 13 mmol/l were recruited into the study. On day 0, mice commenced drug treatments for 28 days as follows: Group 1 (HF control) – high fat mice administered saline vehicle (0.9% wt/vol; p.o.; o.d.); Group 2 (dapagliflozin) – high fat mice administered dapagliflozin (1 mg/kg; p.o.; o.d.; Selleck Chemicals; Stratech Scientific Ltd., Suffolk, UK; Catalog number S1548-SEL); Group 3 (DAPA-Lira) – high fat mice administered dapagliflozin (1 mg/kg; p.o.; o.d.) plus liraglutide (25 nmol/kg; i.p.; o.d.; GL Biochem Ltd.,

Shanghai, China); Group 4 (Lira) - high fat mice administered liraglutide (25 nmol/kg; i.p.; o.d.); Group 5 (lean control) – lean mice administered saline vehicle (0.9% wt/vol; p.o.; o.d.). All treatments administered at 14:00 h and mice remained on respective diet for study duration. The rationale for choosing 1 mg/kg dapagliflozin (p.o.) and 25 nmol/kg liraglutide (i.p.) in this study was based on previously published literature (Moffett *et al*. 2014, Millar *et al*. 2016). Energy intake, body weight, glucose and insulin concentrations were measured every 3 to 4 days. At the end of the study, glucose tolerance (18 mmol/kg; p.o.; at 10:00 h in 12 h-fasted mice), insulin sensitivity (25 U/kg bovine insulin; i.p.; at 10:00 h in non-fasted mice), novel object recognition task, dual-energy x-ray absorptiometry (DEXA) scanning, lipids, hormones/biomarkers, islet and brain histology were performed.

Biochemical and DEXA analyses

Blood samples were collected as indicated in Figures from tail vein of conscious mice into chilled fluoride/heparin micro-centrifuge tubes (Sarstedt, Numbrecht, Germany) and centrifuged at 13,000g for 30 s (Beckman Instruments, Galway, Ireland). Glucose concentrations were measured using Ascencia Contour Blood Glucose Meter (Bayer Healthcare, Newbury, UK) and plasma/pancreatic insulin determined using modified dextran-coated charcoal RIA (Flatt & Bailey 1981). HOMA-IR and HOMA-β were determined from calculations as described previously (Gault *et al*. 2015). Lipids (total-cholesterol – CH200; and triglycerides – TR210) and ALT (AL1205) were measured using enzymatic kits from Randox Laboratories (Crumlin, UK). Plasma corticosterone (ab100712) and IL-6 (ab108821) were measured using enzymatic kits from Abcam (Cambridge, UK) and analysed with SOFTMAX PRO Software Version 5.2 on Flexstation 3 (Molecular Devices, Sunnyvale, CA, USA). Glucagon and total GLP-1 were measured by ELISA (EZGLU-30K and EZGLP1T-36K, respectively; Millipore, UK). HbA1c was determined with a commercially available kit (HB-3058;

Chirus Limited, Watford, UK). Percentage fat and lean mass were measured using DEXA densitometry (Piximus Densitometer, USA) as described previously (Millar *et al*. 2016).

Assessment of learning and memory

Open field and novel recognition tests were performed as described previously (Lennox *et al*. 2014). Briefly, mice were placed in an arena and motor activity (speed and path length), anxiety (grooming events) and exploration (rearing events) recorded over 5 min period. Twenty-four hours later, mice were placed back into the same arena and a novel object recognition task was conducted comprising a 10 min acquisition phase (followed by a 3 h rest in the home cage) followed by test trial where mice could explore familiar and novel object for 10 min. Time spent exploring familiar or novel object was expressed as recognition index (RI) calculated as time (t) spent exploring novel object divided by time spent exploring both objects (A + B) x 10. $RI_B = tB/t(A + B) \times 100$ normalises all data for statistical comparison (Lennox *et al*. 2014).

Immunohistochemistry and image analysis

Mice were perfused with PBS transcardially as described previously (Parthsarathy *et al*. 2013). Pancreatic tissue was excised for immunohistochemistry, measurement of insulin/glucagon content and gene expression. For determination of pancreatic insulin and glucagon content, pre-weighed pancreatic tissue was washed thoroughly in ice-cold PBS, homogenised in acid ethanol solution (ethanol/0.7 M HCl; 3:1 ratio) and extracted overnight at 4°C. Insulin content was measured by insulin radioimmunoassay and glucagon content determined by ELISA (EZGLU-30K; Millipore, UK). For histology, pancreatic tissues were fixed in 4% paraformaldehyde for 48 h at 4°C, processed using automated tissue processor (Leica TP1020, Leica Microsystems, Nussloch, Germany) and embedded in paraffin wax. Immunohistochemistry was performed as described

previously (Moffett *et al*. 2015). Following primary antibodies used: mouse monoclonal anti-insulin antibody (ab6995, 1:1000; Abcam), guniea-pig anti-glucagon antibody (PCA2/4, 1:400; raised in-house), rabbit polyclonal anti-GLP-1 antibody (XJIC8, 1:200; raised in-house) and mouse polyclonal anti-IL-6 details (PM626, 1:200; ThermoFisher Scientific). Secondary antibodies used as appropriate: Alexa Fluor 488 goat anti-guinea pig IgG – 1:400, Alexa Fluor 594 goat anti-mouse IgG – 1:400. Slides were viewed under FITC filter (488 nm) or TRITC filter (594 nm) using fluorescent microscope (Olympus BX51) and DP70 camera adapter system. Brain processing and immunostaining were performed as described previously (Parthsarathy *et al*. 2013). Briefly, 40 micron thick coronal sections of brains at anatomical regions -2 to -3 bregma were stained for young immature neurons (anti-doublecortin, 1:200 dilution, sc-8066, Santa Cruz Biotechnology) and synaptic density (anti-synaptophysin, 1:200 dilution, Abcam, ab-7837).

Image analysis

Alpha and beta cell area were analysed in a blinded manner using Cell^F image analysis software (Olympus Soft Imaging Solutions, GmbH) and expressed as μm^2. Briefly, fluorescent images were captured using digital camera and closed polygon tool in Cell^F used to analyse alpha cell and beta cell area. Pixel area was converted to μm^2 and plotted in Prism. To quantify cell proliferation and neurogenesis, DCX-labelled immature neurons were counted in sub granular zone of dentate gyrus. Minimum of seven coronal sections per animal were counted using 40 x objective of bright field microscope (Olympus BX51) and plotted as average number of positive cells per section. Synaptophysin staining was analysed with Image J (NIH, USA) software using corrected O.D. method (McClean *et al*. 2011). Briefly, following adjustment for optimum resolution, calibration for optical density was performed using Kodak No. 3 step tablet (Tiffen, Kodak) and calibration curve obtained as described in Image J software. Using 10 x magnification objective, image for each area

of interest was obtained per section (4-5 sections per mouse brain) with digital camera. Area of interest comprised hippocampus and cortex that included polymorphic layer, granular cell layer, molecular layer, stratum radiatum, stratum pyramidal, stratum oriens, interior and exterior cortical layers. Images were converted to 8-bit grey scale and pixel density obtained from three small randomly selected squares per layer converted to O.D. using calibration curve. Average O.D values for each layer were subtracted from average O.D values of granular cell layer (GCL) and corrected O.D. plotted.

Gene expression

mRNA extracted (Tripure Isolation Reagent; Roche Diagnostics, UK), quantified and purity determined using nanophotometer (Implen, Munich, Germany). cDNA synthesized using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics) and gene expression analysis for insulin and glucagon performed on whole pancreas by qPCR using Light Cycler 480 Probes Master (Roche Diagnostics) according to manufacturer's instructions (Gault *et al*. 2015). HPRT and beta-actin were used as internal control for normalisation. PCR conditions were 95ºC for 10 min, followed by cDNA amplification for 50 cycles with 95ºC denaturation for 10s, 60ºC annealing for 30 s and 72ºC elongation for 10s followed by cooling period of 30 s at 40 °C. Relative quantification using $2^{-\Delta\Delta CT}$ method used to calculate differences between groups (Livak & Schmittgen 2001).

Statistical analyses

Results were analysed using Prism (GraphPad Software Inc., USA) and data expressed as mean ± S.E.M. For metabolic data, statistical analyses were performed using one-way ANOVA followed by Student-Newman-Keuls *post-hoc* test. For novel object recognition and immunohistochemistry, statistical analyses were carried out using unpaired Student's t-test (non-parametric, with two-tailed

217 *p* values and 95% confidence interval) and one-way ANOVA with Bonferroni *post-hoc* test. Groups
218 of data were considered to be significantly different if $p<0.05$.
219
220 **Results**
221 **Effects of DAPA-Lira on body weight, energy intake, glucose and insulin concentrations**
222 Compared to HF controls, DAPA-Lira treatment resulted in significant time-dependent decrease in
223 body weight ($p<0.001$; Fig. 1A). Importantly, body weights for DAPA-Lira and dapagliflozin
224 groups were reduced despite no reduction in energy intake (Fig. 1B). Liraglutide-treated mice
225 displayed reduced cumulative energy intake ($p<0.05$-$p<0.001$; Fig. 1B) compared to HF controls.
226 DAPA-Lira treatment resulted in time-dependent decrease (242%; $p<0.001$) in glucose
227 concentrations compared to HF controls, dapagliflozin or liraglutide alone (157-172%; $p<0.01$) on
228 day 28 (Fig. 1C). All treatments exhibited progressive time-dependent increase in insulin but no
229 significance was detected between HF groups, except DAPA-Lira treated mice which exhibited
230 lower levels on day 28 ($p<0.01$; Fig. 1D).
231
232 **Effects of DAPA-Lira on glucose tolerance, insulin response to glucose, insulin sensitivity,**
233 **HbA1c and plasma glucagon**
234 Mice treated with DAPA-Lira for 28 days exhibited significant reduction (37-47% decrease; $p<0.01$)
235 in glucose concentrations (individual time-course for up to 120 min) compared with dapagliflozin or
236 liraglutide alone (Fig. 2A). This was further corroborated by significantly reduced glucose AUC_{120}
237 values (37-52% decrease; $p<0.001$; Fig. 2B). As shown in Fig. 2C, all treatment groups
238 demonstrated increased insulinotropic response, with DAPA-Lira mice exhibiting significantly
239 higher AUC_{90} values (1.1-1.4-fold increase; $p<0.01$-$p<0.001$) compared to dapagliflozin or
240 liraglutide alone (Fig. 2D). Similarly, all treatment groups displayed marked improvement in insulin

sensitivity compared to HF controls following administration of exogenous insulin ($p<0.05$; Fig. 3A-B). DAPA-Lira treated mice also displayed marked reduction (73% lower; $p<0.05$) in HOMA-IR compared to dapagliflozin or liraglutide alone (Fig. 3C). Furthermore, mice treated with DAPA-Lira had a significantly improved HOMA-β index compared to dapagliflozin (53% increase; $p<0.01$) or liraglutide (13% increase; $p<0.05$) alone (Fig. 3D). All treatment groups had significantly ($p<0.001$) reduced HbA1c values compared to HF controls with DAPA-Lira treated mice exhibiting improved HbA1c (19-26% reduction; $p<0.001$) compared to dapagliflozin or liraglutide alone (Fig. 3E). Dapagliflozin and DAPA-Lira groups had significantly increased plasma glucagon concentrations (24-33%; $p <0.001$) compared to HF controls, whereas liraglutide group exerted a 33% reduction in plasma glucagon ($p<0.001$) compared to DAPA-Lira treated mice (Fig. 3F).

Effects of DAPA-Lira on body composition and lipids

DEXA analysis revealed that all treatment groups exhibited significant reduction (37-42% decrease; $p<0.05$-$p<0.001$) in percentage fat mass compared to HF controls (Fig. 4A). No significant differences were noted between DAPA-Lira and liraglutide or dapagliflozin. Similarly, no significant differences were observed in lean mass for any groups tested (Fig. 4B). Compared with HF controls, all treatments significantly decreased triglycerides ($p<0.5$-$p<0.001$; Fig. 4C). DAPA-Lira reduced triglycerides (71-87% decrease; $p<0.01$-$p<0.001$; Fig. 4C) to a greater extent than either liraglutide or dapagliflozin alone. No significant differences between HF groups in terms of total cholesterol was observed (Fig. 4D).

Effects of DAPA-Lira on terminal organ weight, hormones and biomarkers

Administration of DAPA-Lira and dapagliflozin resulted in a significant reduction in inguinal adipose weight ($p<0.05$; Fig. 5A). Liver and pancreatic weights were not significantly different in

any of the HF groups (Fig. 5B and 5C). Total plasma GLP-1 concentrations were significantly increased in all treatment groups ($p<0.001$) compared to HF controls with DAPA-Lira group displaying increased levels of total GLP-1 (12-18% increase; $p<0.01$-$p<0.001$) compared to liraglutide or dapagliflozin alone (Fig. 5D). No significant differences were noted in ALT levels in HF mice (Fig. 5E). Both dapagliflozin and DAPA-Lira treatment groups displayed significantly elevated (1.0-fold; $p<0.01$-$p<0.001$) plasma IL-6 levels compared to HF controls (Fig. 5F). Liraglutide only treated mice displayed reduced (48% decrease; $p<0.001$) IL-6 levels compared to DAPA-Lira treatment (Fig. 5F). All treatment groups resulted in significant reduction (33-43% decrease; $p<0.05$) in corticosterone concentrations compared to HF controls (Fig. 5G).

Effects of DAPA-Lira in novel object recognition task

During test trial, no significant difference was noted in the recognition index (RI) for the HF group indicating that they could not discriminate between novel and familiar object thereby exhibiting impaired cognition (Fig. 6A). All treatment groups and lean control group displayed significantly increased RI (1.1-1.3-fold; $p<0.01$-$p<0.001$) when exposed to novel object compared to HF controls, thus highlighting preference to explore novel *versus* familiar object (Fig. 6B-6F). Open field assessment revealed no effect of any treatments on motor activity (speed and path length), anxiety (grooming events) and exploration (rearing events) (data not shown).

Effects of DAPA-Lira on islet morphology, pancreatic hormone content and mRNA gene expression

As shown in Fig. 7A, beta cell area was significantly increased ($p<0.05$) in liraglutide treated mice. Mice treated with dapagliflozin alone exhibited marked increase ($p<0.01$) in alpha cell area compared to HF controls (Fig. 7D). In contrast, DAPA-Lira or liraglutide alone did not affect alpha

cell area (Fig. 7D). Islets of HF mice exhibited substantial staining for IL-6 in beta cells and GLP-1 in alpha cells with no appreciable differences between various treatment groups (images not shown). Both DAPA-Lira and liraglutide markedly increased (1.4-1.6 fold; $p<0.05$) pancreatic insulin content compared to HF controls (Fig. 7B). Liraglutide treatment also significantly decreased (23% reduction; $p<0.001$) glucagon content while both DAPA-Lira and dapagliflozin led to significant increases compared to HF controls (21-28% increase; $p<0.01$-$p<0.001$; Fig. 7E). A similar pattern to changes in pancreatic insulin and glucagon content were observed in pancreatic mRNA expression of insulin and glucagon (Fig. 7C and 7F).

Effects of DAPA-Lira on neurogenesis and synaptic density

Representative micrographs of doublecortin and synaptophysin staining are shown in Fig. 8A-8E. Mice treated with DAPA-Lira, dapagliflozin or liraglutide displayed increased number of immature neurons in the dentate gyrus (44-69% increase; $p<0.01$-$p<0.001$; Fig. 8F) compared to HF controls as indicated by increased number of DCX-positive cells. Significantly higher levels of synaptophysin expression were demonstrated in all treatment groups in stratum oriens layer (88-113% increase; $p<0.01$; Fig. 8J) compared to HF controls. DAPA-Lira treatment also improved synaptophysin expression in stratum pyramidale layer (50% increase; $p<0.05$; Fig. 8I), though no differences in polymorph layer (Fig. 8G) and stratum radiatum (Fig. 8H) were observed.

Discussion

Given the increase and diversity of new antidiabetic drugs in the clinic, there is now a great opportunity to offer a more patient-centered tailored or personalized approach to therapeutic intervention. In this study, we examined the efficacy of combination therapy using the SGLT2 inhibitor dapagliflozin and the long-acting GLP-1 agonist liraglutide. In addition to assessing

313 metabolic outcomes, we also examined potential neuroprotective benefits of combination therapy on
314 learning and memory, especially since recent evidence has shown that GLP-1 agonists may reduce
315 cognitive decline in diabetes-obesity (Gault *et al*. 2010, Porter *et al*. 2013).

316 We chose to use a rodent model of diabetes combining low-dose STZ and high fat feeding to
317 promote obesity, insulin resistance and hyperglycaemia (Srinivasan *et al*. 2005, Islam & Wilson
318 2012). This rodent model has been used previously and serves as a suitable means to evaluate
319 potential drug intervention (Bhat *et al*. 2013, Millar *et al*. 2016). A small dose of STZ was combined
320 with high fat feeding to inflict sub-lethal damage to beta cells which when combined with high fat
321 feeding gave a more rapid and pronounced diabetes phenotype with elevation in blood glucose
322 concentrations. SGLT2 inhibitors act by reabsorbing glucose so it is useful to study therapeutic
323 effects when glucose levels are significantly raised. In contrast to liraglutide, DAPA-Lira
324 combination therapy over 28 days did not affect energy intake. This is an important observation as
325 several studies suggested that energy intake is increased following SGLT2 inhibitor therapy
326 (Devenny *et al*. 2012, Nagata *et al*. 2013). As expected, all treatments resulted in reduced body
327 weight, which in the case of dapagliflozin most presumably reflects energy loss via urinary glucose
328 excretion (Scheen & Paquot 2014). SGLT2 inhibitors induce a significant and durable weight loss in
329 patients with T2DM (Bailey *et al*. 2015).

330 Consistent with previous studies, monotherapy with dapagliflozin or liraglutide decreased
331 glucose concentrations both in terms of non-fasting concentrations and following an oral glucose
332 challenge. Moreover, DAPA-Lira combination therapy resulted in a more pronounced glucose-
333 lowering effect that is most likely achieved through increased urinary glucose excretion (Bailey *et*
334 *al*. 2016) and enhanced beta cell function. Interestingly, dapagliflozin monotherapy was also
335 associated with enhanced glucose-induced insulin secretion and HOMA-β, which could be due to
336 improved metabolic control and reversal of beta cell glucotoxicity, a potential direct effect on beta

cells and possible involvement of effects on other hormones such as GLP-1 as observed with less selective SGLT2 inhibitors (Zambrowicz *et al*. 2013). Indeed, plasma GLP-1 concentrations were significantly increased in all treatment groups at the end of the study but most notably in the group receiving DAPA-Lira combination therapy. This may well point to enhanced alpha cell GLP-1 production, which has been observed previously in pregnancy and situations of beta cell stress (Moffett *et al*. 2014, Vasu *et al*. 2014). Interestingly, IL-6 which has been implicated in islet processing of proglucagon to GLP-1 via increased expression of PC1/3 (Ellingsgaard *et al*. 2011) was markedly increased in beta cells of all HF groups.

All treatment groups exhibited improved insulin sensitivity and improved HOMA-IR. These changes in insulin sensitivity may be ascribed to weight reduction and alleviation of glucose toxicity (Macdonald *et al*. 2010). Of particular note is the observation that DAPA-Lira combination therapy markedly reduced HbA1c, which was significantly lower than either dapagliflozin or liraglutide alone. Importantly, no episodes of hypoglycaemia were observed in treatment groups following fasting for OGTT or during the insulin tolerance test, however measurement of circulating ketones would have been informative. Taken together, DAPA-Lira combination therapy was associated with improved glucose-lowering and greater reduction in body weight (compared to liraglutide alone), without observable effects on energy intake, suggesting that combination of dapagliflozin and liraglutide is a very powerful approach to management of glycaemia.

Consistent with previous studies, high fat fed mice exhibited dyslipidaemia and obesity (Podrini *et al*. 2013). DEXA scanning revealed that fat mass was significantly reduced in all treatment groups with tendency to be lower in groups treated with dapagliflozin and this was further corroborated by significantly decreased adipose tissue mass. In T2DM patients, body weight loss induced by dapagliflozin is mainly due to reduction in visceral and subcutaneous fat mass (Bolinder *et al*. 2012). Importantly, decreases in fat mass were not associated with changes in lean mass. High

fat mice displayed elevated triglyceride concentrations that were significantly improved in all treatment groups but especially in DAPA-Lira combination group. This could be due to function of improved glycaemia and greater weight reduction in this group. No significant changes in plasma total cholesterol were noted in any of the treatment groups. Although not measured in this study, relatively small clinically insignificant increases in both LDL- and HDL-cholesterol have been observed in patients on dapagliflozin therapy (Matthaei *et al*. 2015). As expected, HF mice exhibited increased liver weight and ALT concentrations characteristic of non-alcoholic fatty liver disease (Ganz *et al*. 2014). Whilst all treatments tended to reverse negative effects towards that of healthy controls, more detailed analyses investigating effects on hepatic triglyceride content, plasma and liver oxidative stress would be useful.

Mice treated with dapagliflozin, either alone or in combination with liraglutide, displayed elevated levels of IL-6. IL-6 has been shown to stimulate insulin from beta cells, glucagon from alpha cells and GLP-1 secretion from both intestinal L and pancreatic alpha cells (Ehses *et al*. 2007, Ellingsgaard *et al*. 2008, 2011). Increase in GLP-1 production is thought to occur via differentiation of the alpha cell through proglucagon transcription and enhanced PC1/3 expression (Ellingsgaard *et al*. 2008, 2011). Furthermore, SGLT2 inhibition promotes glucagon secretion from alpha cells in healthy mice (Bonner *et al*. 2015) and increases GLP-1 concentrations in patients (Ferrannini *et al*. 2014). More recent studies have suggested that dapagliflozin stimulates GLP-1 and IL6 secretion from pancreatic islets (Timper *et al*. 2016). In the present study, dapagliflozin increased plasma GLP-1, glucagon and IL-6, perhaps pointing to an important role for IL6 in beneficial action of dapagliflozin. Indeed, we have recently demonstrated increased expression of PC1/3 in αTC1.9 cells treated with dapagliflozin (unpublished observations). Measures of additional circulating cytokines (e.g. TNFα and IL-1β) would provide insight as to whether this reflects a specific effect on IL-6, or a more generalized heightened inflammatory state. The increase in plasma IL-6 following

dapagliflozin therapy is particularly interesting and further studies exploring its role are clearly warranted. Importantly, mice treated with liraglutide alone displayed decreased plasma glucagon with no change in IL-6 concentrations.

Pancreatic immunochemical staining revealed that mice treated with dapagliflozin exhibited a significant increase in alpha cell area. This was accompanied by reduction in pancreatic insulin content and increases in both proglucagon gene expression and pancreatic glucagon content. These observations are broadly in line with metabolic insulin and glucagon data and further confirm an important effect of dapagliflozin on the alpha cell (Bonner et al. 2015). As expected, liraglutide treatment was associated with enhanced beta cell area and increased insulin content and this was accompanied by significant decrease in gene expression and pancreatic glucagon content (Schwasinger-Schmidt *et al*. 2013). Interestingly, alpha cell area was not affected in mice receiving DAPA-Lira combination therapy with both insulin gene expression and hormone content increased, suggesting that the liraglutide component countered some of the alpha cell promoting properties of dapagliflozin, which would be beneficial in a longer-term treatment regimen. We did not see tight correlation between the various parameters (percentage islet cells, hormone content and basal hormone levels) because many different factors influence these parameters. For example, if cell synthesizes hormone it is not just leaked out into the blood but is stored in vesicles that are regulated on minute-to-minute basis by prevailing blood glucose plus myriad of other factors.

We and others have previously shown that high fat feeding in rodents causes detrimental effects in brain regions associated with learning and memory (Greenwood & Winocur 2005, Stranahan *et al*. 2008a, Gault *et al*. 2010). Furthermore, growing evidence indicates that diabetes and obesity increase the risk of developing neurodegenerative disorders, such as Alzheimer's disease (Rani *et al*. 2016). More recently, GLP-1 agonists (and DPP4 inhibitors) have been shown to reduce cognitive decline associated with diabetes-obesity (Groeneveld *et al*. 2016). The present

study evaluated learning and memory using well-established novel object recognition task, which exploits ability of a rodent to explore a novel object over a familiar object (Abbas *et al*. 2009). As expected, HF mice could not discriminate between familiar and novel object when compared to healthy controls (Gault *et al*. 2015). However, all HF treated mice displayed significantly improved recognition memory, which was not attributable to effects on anxiety or motor activity, as indicated in open field assessment. Future studies using additional behavioural tests such as Morris Water Maze would also be useful.

Corticosterone concentrations were markedly raised following high fat feeding but treatment with dapagliflozin or liraglutide (both alone and in combination) reversed this effect. Raised glucocorticoid concentrations not only induce insulin resistance but also contribute to deficits in hippocampal function (Stranahan *et al*. 2008b). Indeed, reducing corticosterone concentrations can prevent diabetes-induced impairment of hippocampus-dependent learning (Stranahan *et al*. 2008c). Immunohistochemical staining revealed that HF treated mice displayed significantly enhanced doublecortin and synaptophysin expression indicating possible role of drug treatment to promote recovery of neurogenesis and synaptic density. It is possible that combination therapy over a longer time period may have resulted in superior neurogenesis and cognitive function compared to monotherapy. To our knowledge, this is the first study to report beneficial effects of dapagliflozin on cognitive function, neurogenesis, and synaptic density. Whilst positive neuroprotective and growth factor like effects of dapagliflozin on learning and memory are unlikely to occur as direct effect of SGLT2 inhibition in the brain itself, SGLT2 inhibitors are lipid-soluble and should cross the blood-brain-barrier (Bakris *et al*. 2009). However, similar to DPP4 inhibitors, it is plausible that neuroprotective effects observed for dapagliflozin could be attributed to increased GLP-1 concentrations which can then cross the blood-brain-barrier and/or actions of dapagliflozin to lower corticosterone concentrations. Whilst we cannot rule out that part of the neuroprotective effects may

be dependent on improved peripheral glycaemia, further detailed studies to delineate mechanism for this improvement in cognitive function with dapagliflozin would be useful.

In summary, compared with either agent alone, DAPA-Lira combination therapy was associated with superior glucose-lowering and significant reduction in body weight, indicating powerful and complementary approach to effectively manage hyperglycaemia. Part of this benefit appears to derive from the ability of liraglutide to decrease islet alpha cells. Other prominent effects included normalisation of hypertriglyceridaemia and enhancements of both insulin secretion and action. Furthermore, DAPA-Lira combination therapy, and indeed dapagliflozin monotherapy, were effective in reversing memory impairment in diabetic mice. Moreover, changes in glucagon and GLP-1 following dapagliflozin treatment were associated with changes in IL-6, suggesting possible role of IL-6 in mediating some of the actions of SGLT2 inhibition. This study supports recent papers showing clinical effectiveness of combination therapy with SGLT2 inhibition and stable GLP-1 mimetics in T2DM patients (DeFronzo 2017). Taken together, our results highlight an important personalised approach in utilising liraglutide in combination with dapagliflozin for further clinical evaluation in the treatment of diabetes and associated neurodegenerative disorders.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contributions

PJBM contributed to study design, conduct/data collection, data analysis and writing of the manuscript. VP, RCM, VParth and NMP contributed to conduct/data collection and data analysis. AJB and MO'K reviewed the manuscript. VAG and PRF contributed to study design, analysis and writing of the manuscript. All authors approved the final version of the manuscript.

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Figure legends

Figure 1: Effects of once-daily administration of DAPA-Lira on (A) body weight, (B) cumulative energy intake, (C) glucose and (D) insulin concentrations. HF mice received saline vehicle (0.9% wt/vol; p.o.), dapagliflozin (1 mg/kg; p.o.), dapagliflozin (1 mg/kg; p.o.) plus liraglutide (25 nmol/kg; i.p) or liraglutide alone (25 nmol/kg; i.p) over 28 days. Lean control mice received saline vehicle once-daily. Metabolic parameters were measured every 3 to 4 days. Values are means ± SEM for groups of 8-10 mice. *p<0.05, **p<0.01 and ***p<0.001 compared to HF controls. $^{\Delta\Delta}p$<0.01 compared to DAPA-Lira.

Figure 2: Effects of once-daily administration of DAPA-Lira on (A and B) glucose tolerance and (C and D) insulin response to glucose. HF mice received saline vehicle (0.9% wt/vol; p.o.), dapagliflozin (1 mg/kg; p.o.), dapagliflozin (1 mg/kg; p.o.) plus liraglutide (25 nmol/kg; i.p) or liraglutide alone (25 nmol/kg; i.p) over 28 days. Lean control mice received saline vehicle once-daily. Glucose and insulin concentrations were measured prior to and after oral administration of glucose (18 mmol/kg) in 12-hour fasted mice. Glucose and insulin AUC values post-injection are also shown. Values are means ± SEM for groups of 8-10 mice. *p<0.05, **p<0.01 and ***p<0.001 compared to HF controls. $^{\Delta\Delta}p$<0.01 and $^{\Delta\Delta\Delta}p$<0.001 compared to DAPA-Lira.

Figure 3: Effects of once-daily administration of DAPA-Lira on (A and B) insulin sensitivity, (C) HOMA-IR, (D) HOMA-β, (E) HbA1c and (F) plasma glucagon. HF mice received saline vehicle (0.9% wt/vol; p.o.), dapagliflozin (1 mg/kg; p.o.), dapagliflozin (1 mg/kg; p.o.) plus liraglutide (25 nmol/kg; i.p) or liraglutide alone (25 nmol/kg; i.p) over 28 days. Lean control mice received saline vehicle once-daily. Parameters were measured at the end of the study period. For insulin sensitivity, glucose concentrations were measured prior to and after injection of insulin (25 U/kg; i.p.) in non-

fasted mice. Glucose AAC values post-injection are also shown. Values are means ± SEM for groups of 8-10 mice. **p<0.01 and ***p<0.001 compared to HF controls. $^{\Delta}p$<0.05, $^{\Delta\Delta}p$<0.01 and $^{\Delta\Delta\Delta}p$<0.001 compared to DAPA-Lira.

Figure 4: Effects of once-daily administration of DAPA-Lira on (A) fat mass, (B) lean mass, (C) triglycerides and (D) total-cholesterol. HF mice received saline vehicle (0.9% wt/vol; p.o.), dapagliflozin (1 mg/kg; p.o.), dapagliflozin (1 mg/kg; p.o.) plus liraglutide (25 nmol/kg; i.p) or liraglutide alone (25 nmol/kg; i.p) over 28 days. Lean control mice received saline vehicle once-daily. Parameters were measured at the end of the study period. Values are means ± SEM for groups of 8-10 mice. *p<0.05 and ***p<0.001 compared to HF controls. $^{\Delta\Delta}p$<0.01 and $^{\Delta\Delta\Delta}p$<0.001 compared to DAPA-Lira.

Figure 5: Effects of once-daily administration of DAPA-Lira on (A) adipose weight, (B) liver weight, (C) pancreatic weight, (D) total GLP-1, (E) ALT, (F) corticosterone, and (G) IL-6 concentrations. HF mice received saline vehicle (0.9% wt/vol; p.o.), dapagliflozin (1 mg/kg; p.o.), dapagliflozin (1 mg/kg; p.o.) plus liraglutide (25 nmol/kg; i.p) or liraglutide alone (25 nmol/kg; i.p) over 28 days. Lean control mice received saline vehicle once-daily. Parameters were measured at the end of the study period. Values are means ± SEM for groups of 8-10 mice. *p<0.05, **p<0.01 and ***p<0.001 compared to HF controls. $^{\Delta\Delta}p$<0.01 and $^{\Delta\Delta\Delta}p$<0.001 compared to DAPA-Lira.

Figure 6: Effects of once-daily administration of DAPA-Lira on recognition index for (A) HF, (B) DAPA, (C) DAPA-Lira, (D) Lira, (E) lean mice and (F) difference score. HF mice received saline vehicle (0.9% wt/vol; p.o.), dapagliflozin (1 mg/kg; p.o.), dapagliflozin (1 mg/kg; p.o.) plus liraglutide (25 nmol/kg; i.p) or liraglutide alone (25 nmol/kg; i.p) over 28 days. Lean control mice

received saline vehicle once-daily. Parameters were measured at the end of the study period. Values are means ± SEM for groups of 8-10 mice. *p<0.05, **p<0.01 and ***p<0.001 compared to HF controls. $^{\Delta}p$<0.05 compared to DAPA-Lira.

Figure 7: Effects of once-daily administration of DAPA-Lira on (A) beta cell area, (B) insulin content, (C) insulin mRNA expression, (D) alpha cell area, (E) glucagon content and (F) glucagon mRNA expression. HF mice received saline vehicle (0.9% wt/vol; p.o.), dapagliflozin (1 mg/kg; p.o.), dapagliflozin (1 mg/kg; p.o.) plus liraglutide (25 nmol/kg; i.p) or liraglutide alone (25 nmol/kg; i.p) over 28 days. Lean control mice received saline vehicle once-daily. Parameters were measured at the end of the study period. Values are means ± SEM for groups of 8-10 mice. *p<0.05, **p<0.01 and ***p<0.001 compared to HF controls. $^{\Delta\Delta}p$<0.01 and $^{\Delta\Delta\Delta}p$<0.001 compared to DAPA-Lira.

Figure 8: Effects of once-daily administration of DAPA-Lira on (A-E) brain immunohistochemistry, (F) doublecortin neuroblast, and quantification levels of synaptophysin expression in (G) polymorph layer, (H) stratum radiatum, (I) stratum pyramidale and (J) stratum oriens layer. HF mice received saline vehicle (0.9% wt/vol; p.o.), dapagliflozin (1 mg/kg; p.o.), dapagliflozin (1 mg/kg; p.o.) plus liraglutide (25 nmol/kg; i.p) or liraglutide alone (25 nmol/kg; i.p) over 28 days. Lean control mice received saline vehicle once-daily. Parameters were measured at the end of the study period. Values are means ± SEM for groups of 6 mice. *p<0.05, **p<0.01 and ***p<0.001 compared to HF controls.

Figure 1

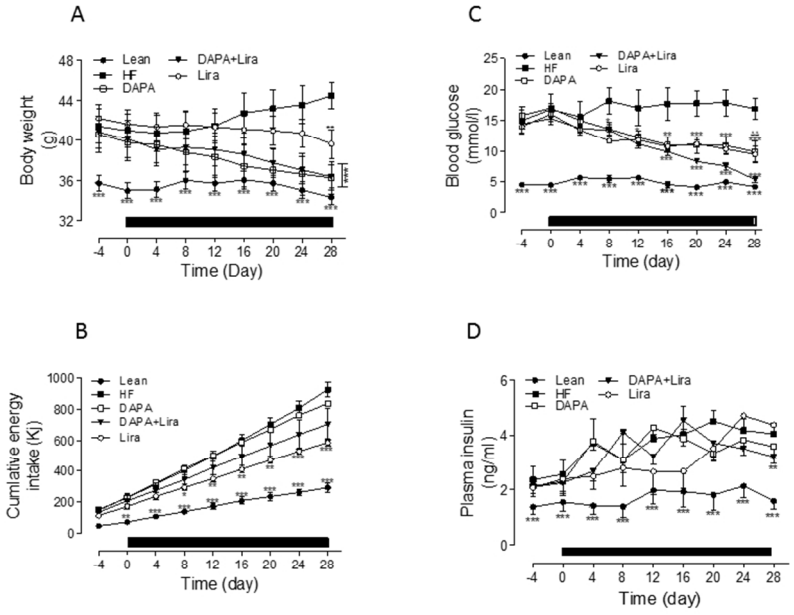

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Figure 2

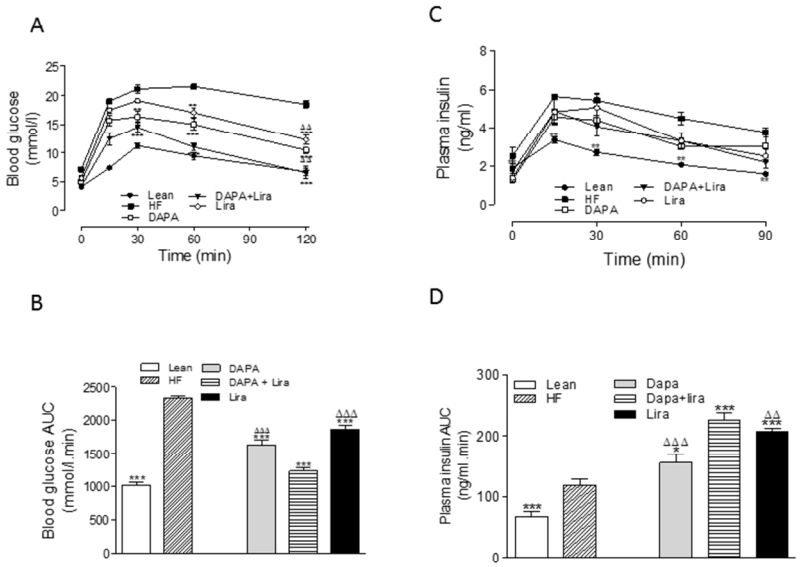

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Figure 3

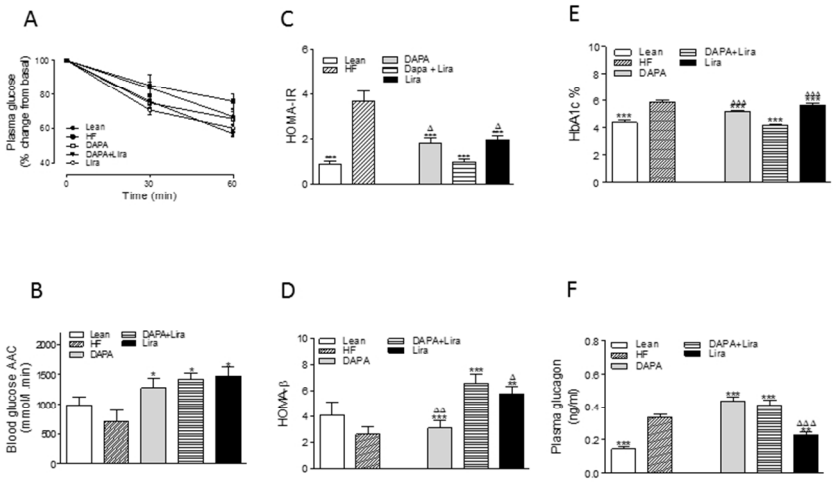

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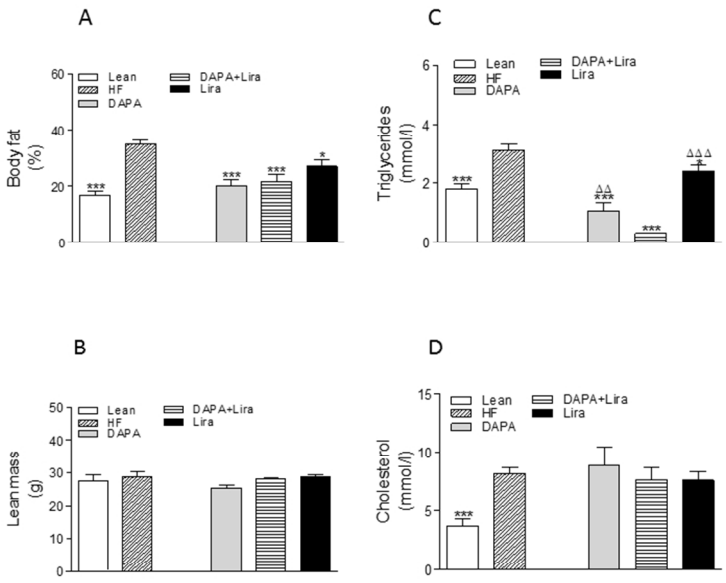
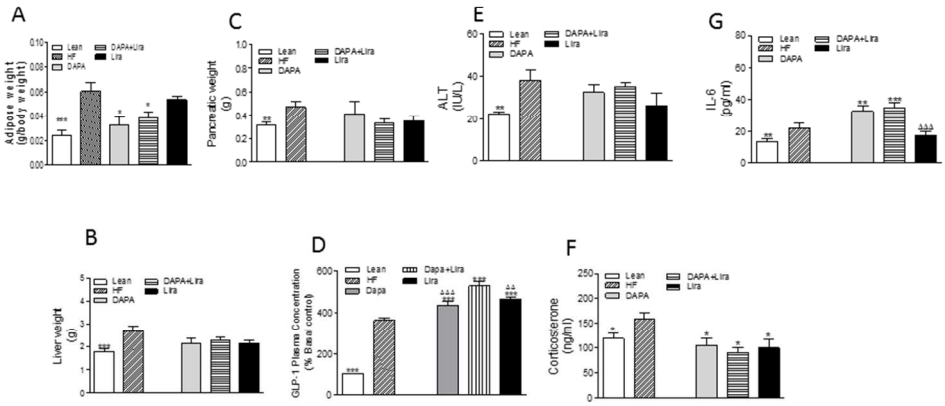
Figure 4



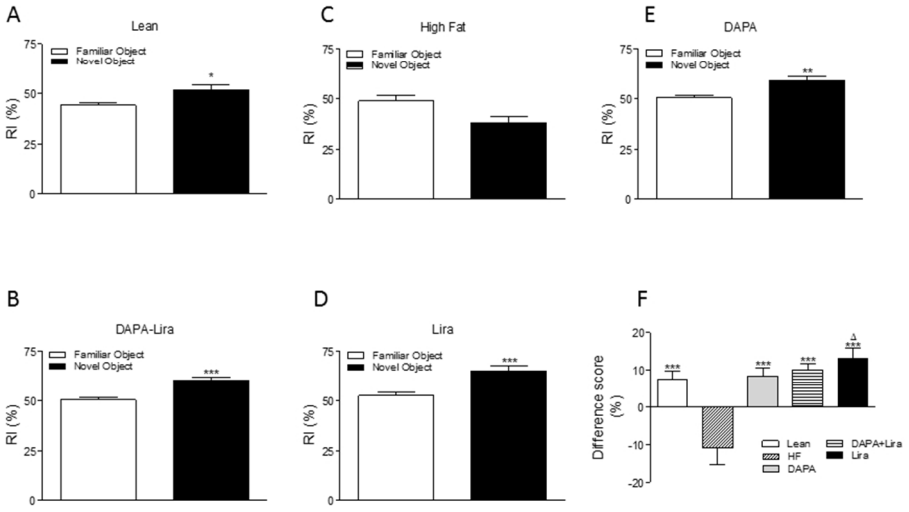
254x190mm (96 x 96 DPI)

Figure 5



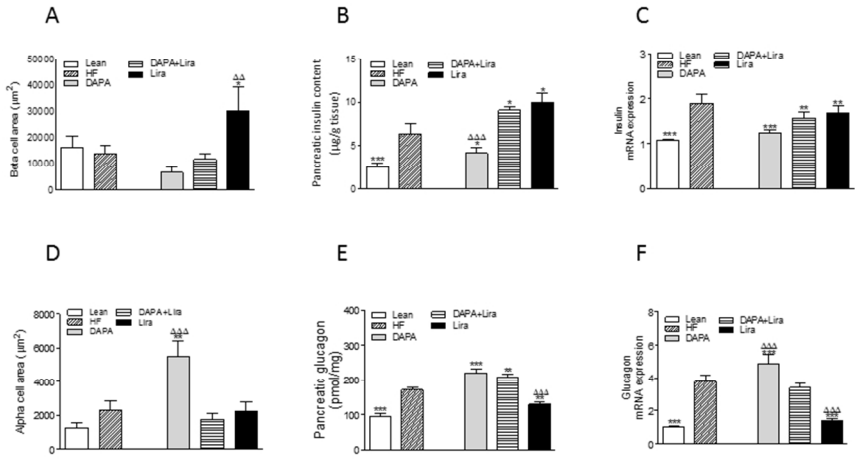
254x190mm (96 x 96 DPI)

Figure 6



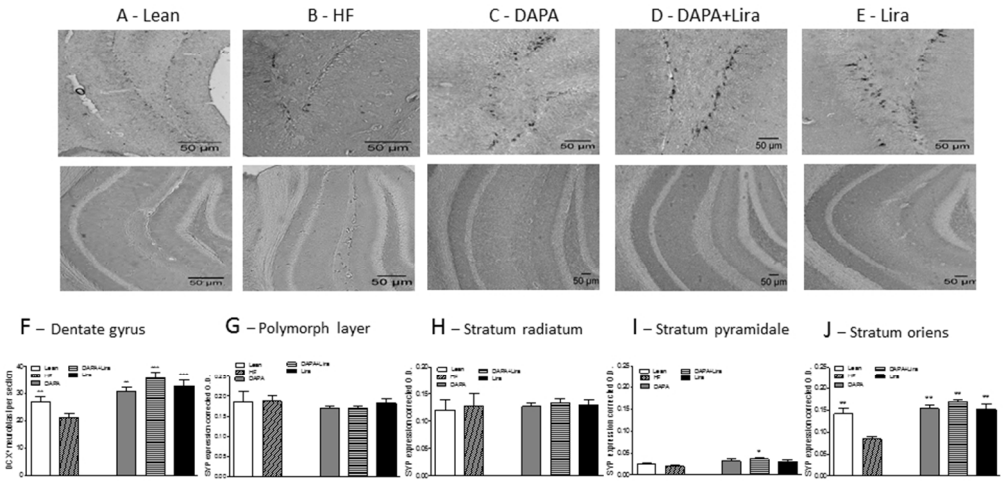
254x190mm (96 x 96 DPI)

Figure 7



254x190mm (96 x 96 DPI)

Figure 8



254x190mm (96 x 96 DPI)