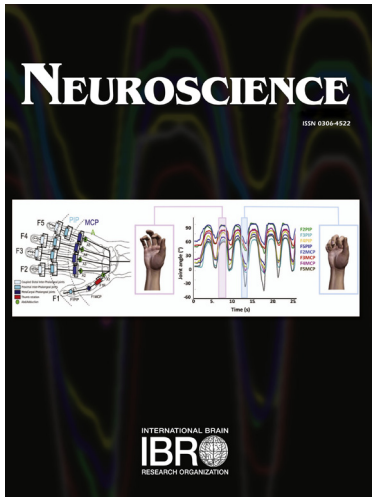

Research Article

The impact of ghrelin on the survival and efficacy of dopaminergic foetal grafts in the 6-OHDA lesioned rat

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The impact of ghrelin on the survival and efficacy of dopaminergic foetal grafts in the 6-OHDA lesioned rat.

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Abstract:

Ghrelin is a peptide produced in the gut with a wide range of physiological functions. Recent studies have suggested it may have potential as a neuroprotective agent in models of Parkinson's disease, reducing the impact of toxic challenges on the survival of nigral dopaminergic neurons. The presence of the ghrelin receptor (GHSR1a) on the dopaminergic neurons of the substantia nigra raises the possibility that a potential application for this property of ghrelin may be as an adjunctive neuroprotective agent to enhance and support the survival and integration of dopaminergic cells transplanted into the striatum. Thus far, inconsistent outcomes in clinical trials for foetal cell transplantation have been linked to low rates of cell survival which we hypothesise could be ameliorated by the presence of ghrelin. To explore this, we confirmed the expression of the GHSR1a and related enzymes on e14 ventral mesencephalon. To determine a functional effect, five groups of female Sprague Dawley rats received a unilateral 6-OHDA lesion to the medial forebrain bundle and four received an intrastriatal graft of e14 ventral mesencephalic cells. Grafted rats received saline; acyl-ghrelin (10 µg/kg); acyl-ghrelin (50 µg/kg) or the ghrelin agonist JMV-2894 (160 µg/kg) i.p. for 8 weeks. An effect of ghrelin at low dose on hippocampal neurogenesis indicated blood brain barrier penetrance and attainment of biologically relevant levels but neither acyl-ghrelin nor JMV-2894 improved graft survival or efficacy.

Introduction:

The motor symptoms of the debilitating, progressive, degenerative condition Parkinson disease (PD) are caused by the loss of nigrostriatal dopaminergic neurons. One therapeutic strategy is the restoration of striatal dopamine through the implantation of dopamine producing cells of embryonic or stem cell origin. This approach has the potential to achieve long lasting relief of motor and non-motor symptoms but is not without significant hurdles. Early double-blind placebo-controlled clinical trials using transplants of foetal ventral mesencephalon produced inconsistent functional benefits for the patients, with the added complexity of the emergence of motor side effects, now commonly known as graft-induced dyskinesia (Freed et al., 2001; Olanow et al., 2003). This variability has since been attributed to factors related to both the donated cells and the patient receiving the grafts (reviewed in (Lane and Smith, 2010; Thompson and Björklund, 2012) One of the biggest challenges in cell transplantation has been the adequate survival of cells following transplantation. Low cell survival rates were reported in human clinical trials (Kordower et al., 1998; Olanow et al., 1996) and this reflects in studies in animal models for whom survival of transplanted dopaminergic neurons varies from 1% to 20% (Brundin et al., 2000). It is not only the number of surviving cells, but also their outgrowth, that is critical to the amount of dopamine produced and the degree of functional recovery that is achieved (Hagell and Brundin, 2001). Transplantation of dopaminergic neurons ectopically into the dopamine denervated striatum makes cells vulnerable to necrosis and apoptosis due to oxidative stress, low trophic factors, excitotoxicity and inflammatory cytokines which ultimately leads to reduced survival post transplantation.

Ghrelin is an acylated hormone consisting of 28 amino acids and is mainly produced in rats by X/A like cells of the gastric mucosa (Date et al., 2000). In the circulation, ghrelin exists in two forms acyl ghrelin and des-acyl ghrelin. Although des-acyl ghrelin represents 90% of total circulating ghrelin, acyl ghrelin is believed to be the essential form for binding with the receptor and producing the physiological action. It influences a wide range of physiological activities, including the stimulation of growth hormone secretion, promotion of appetite and food intake, regulation of energy homeostasis, activation of gastric secretion and motility (Delporte, 2013). It is elevated during calorie restriction, a response unique amongst gastrointestinal hormones but interesting because calorie restriction has been linked to lower predisposition to neurodegenerative disease both in patients and animal models of disease (Maalouf et al., 2009). This neuroprotective activity may derive from a range of possible mechanisms, including anti-apoptotic, anti-oxidant, anti-inflammatory actions as well as boosting mitochondrial function (Morgan et al., 2017). Through raising levels of the uncoupling molecule 2 (UCP2), ghrelin increases the number of mitochondria and improves their respiration levels as well as

widening the cellular buffering capacity to the reactive oxygen species (ROS) during stress (Andrews et al., 2009). These capacities have been demonstrated in both *in vitro* and *in vivo* models of disease. *In vitro* ghrelin can restore mitochondrial membrane potential, reduce ROS production and abolish caspase 3 levels (Dong et al., 2009; Jiang et al., 2008). Similarly *in vivo* ghrelin promotes mRNA expression of Bcl-2, attenuating Bax expression and reducing caspase-3 activity in the substantia nigra of MPTP-treated mice (Jiang et al., 2008) leading to protection of the cells from its toxicity. Reduced malonaldehyde levels following ghrelin administration to MPTP-treated MES23.5 dopaminergic neurons demonstrates it has anti-oxidant activity as well as ability to reverse reductions in Cu–Zn superoxide dismutase (SOD) and catalase (CAT) enzymes (Liu et al., 2010). Moreover, ghrelin subdues harmful inflammatory responses in the MPTP-treated mouse reducing activation of microglia in the substantia nigra and striatum. Ghrelin also decreases activation of inducible nitric oxide synthase and the expression of the pro-inflammatory cytokines (TNF-alpha and IL1β) (Moon et al., 2009). It inhibits the release of IL6, a pro-inflammatory cytokine, from dopaminergic neurons when they are stimulated by lipopolysaccharide and mediates this effect through the IL6 receptor (CD126) on dopaminergic neurons (Beynon et al., 2013).

GHSR1a receptors, the target of acyl ghrelin, have been identified on the dopaminergic neurons of the adult rat substantia nigra (Suda et al., 2018a; Zigman et al., 2006). In this study we hypothesised that if the receptors are present on immature dopaminergic neurons in development, that they may also be a target to increase the survival of transplanted dopaminergic primary cells. This study evaluated whether acyl ghrelin or a long lasting synthetic agonist, JMV-2894, could serve to improve graft survival and functionality in the 6-OHDA lesioned rat model of Parkinson's disease.

Materials and Methods

Adult female Sprague Dawley rats (weighing 190-200g at the start of the experiment) housed in groups of 4-5 and time mated pregnant Wistar rats housed in pairs were obtained from Envigo, housed at 21ºC with humidity of 45-65% and a 12 hour light cycle. They were supplied with hygienic animal bedding with *ad libitum* access to food (14% protein, Envigo) and water. All the experiments were performed in accordance with EU regulation 1986 under Home Office licence PIL reviewed by Cardiff University Animal Welfare and Ethical Regulation Body. Drugs and chemicals: Acyl ghrelin (rat) was obtained from Tocris Bioscience, JMV-2894 from Aeterna Zentaris, 6-OHDA HBr from Sigma Alrich, UK)

Experimental design:

Thirty-two Sprague Dawley rats were stereotactically lesioned by an infusion of 6-OHDA into the medial forebrain bundle. Three weeks later, motor and behavioural deficits were measured by performance in amphetamine-induced rotations (ipsilateral rotations ≥ 6 / min considered a successful lesion with more than 90% DA loss), cylinder, adjusting step and vibrissae tests. Rats were then allocated into 5 balanced groups based on performance in these tasks groups (n=6-7). A week later, four groups underwent allogenic E14 VM cells transplantation in the striatum and with one lesion-only control group. The transplanted groups were administered one of the following treatments: saline; acyl ghrelin (10 µg/kg); acyl ghrelin (50 µg/kg) or the ghrelin agonist JMV-2894 (160 µg/kg). The acyl Ghrelin (rat) used in this experiment was obtained commercially from TOCRIS bioscience (Cat number 1465) while JMV-2894 was obtained from Aeterna Zentaris GmbH. Drug administration commenced on the day of transplantation and continued for 8 weeks. Treatments were given daily, prior to the commencement of the dark phase to coincide with the peak physiological levels of endogenous ghrelin. In addition, the rats had no access to food for 3 hours immediately following drug administration with the aim of decreasing serum glucose levels which may potentiate the protective effect of the ghrelin (Andrews et al. 2009). Motor and behavioural tests were evaluated at 4, 6 and 8 weeks after transplantation. Rats were weighed daily with no effect of drug treatment on body weight compared to saline treated groups (data not shown). Finally, all the rats were perfused and the brains harvested for histological analysis (Figure 1).

Surgical Procedures:

All the surgical procedures were carried out following aseptic surgical techniques. Rats were anaesthetised with 4-5% isoflurane in a carrier of medical oxygen for the induction, then maintained at 2-3% isoflurane and 4% nitrous oxide. The surgeries were performed using a Kopf stereotactic

frame and a Harvard micro-drive infusion pump. 6-OHDA lesion surgery involved infusion of 3 µl of a 6-OHDA solution (25 mM 6-OHDA + 0.025% ascorbic acid, Sigma) into the medial forebrain bundle of the right hemisphere at the following coordinates from bregma AP: - 4mm, ML: - 1.3 mm, DV: - 7 mm below dura with the nose bar set at -4.5mm (Torres et al., 2011; Ungerstedt, 1968). Transplantation surgery involved infusion of E14 VM cell suspension in two deposits at one injection site into the right striatum using coordinates: AP: - 0.5 mm, ML: - 3 mm, DV - 5 mm and - 4 mm and nose bar: - 4.5 mm. 1 µl of cell suspension was infused at a rate of 1 µl/ 90 sec using a 23-gauge stainless steel cannula. At the end of the intervention, the rats were sutured with vicryl 4-0 sutures and received 30 µl Metacam (5 mg/ml) and 5 ml 0.9% saline subcutaneously.

Preparation of ventral mesencephalon:

Embryos were collected from E14 Wistar time-mated pregnant rats with CRL 10.5-11 mm confirming age. VM pieces were dissected in Hanks' balanced salt solution (HBSS) solution. A standard protocol was then followed to make a single cell/small cluster of cells suspension (Björklund et al., 1983; Torres et al., 2007). VM pieces were washed 3 times with Dulbecco's minimum Eagle medium (DMEM) solution followed by incubation in 1.5 ml of TryplE™ express solution with 30 µl of DNase solution at 37 ^{0} C for 20 min. This was followed by three washes of DMEM/DNase solution (14ml DMEM + 280µl DNase). Mechanical dissociation was then applied through trituration. The viability and cell number was checked using a 0.04% trypan blue stain. The cell suspension was centrifuged at a rate of 380 G for 3 min at room temperature and re-suspended in DMEM/DNase to achieve a final concentration of one third of a VM per µl.

Motor and behavioural tests

Amphetamine-induced rotation testing was performed by administration of meth-amphetamine (2.5 mg/kg i.p) and counting the frequency and direction of the rotations in an automated rotameter (Rotarat) (Ungerstedt and Arbuthnott, 1970). Simple hand motor tests (stepping, vibrissae and cylinder) were evaluated by determining the percentage response of the contralateral forelimb compared to the ipsilateral forelimb. In the adjusting step test, rats were moved along 1m of bench over 10 seconds in a forehand direction with the body weight on one forepaw and the rest of the body supported by the handler. Right (ipsilateral) and left (contralateral) forelimb steps were counted (Kirik et al., 1998). In the vibrissae test, the paw placing reflex onto a surface edge in response to a light touch of the whiskers were counted on the ipsilateral and contralateral side (Schallert et al., 2000). In the cylinder test, rats were placed in a Perspex cylinder (height: 33.5 cm, diameter: 19 cm) and the

percentage of weight bearing contralateral paw touches to the cylinder surface out of the first 20 total touches was calculated (Schallert et al., 2000).

Perfusion and Fixation:

Rats were anaesthetized by injecting 0.7 ml of sodium pentobarbital (Euthatal, Merial, UK) 200mg/ml then perfused transcardially with 0.9% phosphate buffered saline followed by 4% paraformaldehyde in 0.01 M PBS solution. Brains were harvested and post-fixed in 4% PFA solution for 4 h then transferred into 25% sucrose solution. Then they were cut using a freezing microtome at 30 µm thickness in a 1:12 series.

Immunohistochemistry

3-3'Diaminobenzedine and double fluorescent immunohistochemistry was applied to free-floating tissue sections. Sections were blocked with 3% serum then incubated with primary antibody in 1% blocking solution overnight at room temperature. Sections were then washed and incubated with biotinylated secondary antibody followed by washing and incubating with either avidin-biotinylated complex and colour produced by incubation with 3-3'diaminobenzedine or streptavidin cy3 and the second primary antibody added following the same procedure. Sections were then mounted on gelatinized slides, dehydrated in graded concentration of alcohol and delipidated with xylene before coverslipping with DPX solution. Primary antibodies used were: Tyrosine hydroxylase TH 1:1000 (Abcam); doublecortin DCX 1:1000 (Abcam) and GHSR1a 1:200 (Alomone Labs). Quantification of TH labelled cells in the striatal graft conducted by counting using 20X objectives Leica light® microscope and corrected with Abercrombie equation ($N=\sum \{n \times F \times T/(T+H)\}$; N = Total corrected number, n= number of the counted cells, F= frequency of the sections (1/12), T= thickness of the sections (30 µm), and H= mean diameter of the cells (Hedreen, 1998)**.** Hippocampal neurogenesis was quantified by labelling neuroblasts in the dentate gyrus for DCX. The labelled cells were counted in the sub granular zone (SGZ) of the dentate gyrus using 20X objectives Olympus B 50 stereology microscope then the cell density within the counted area (granular cell layer plus SGZ) was measured.

Immunocytochemistry:

An E14 VM cell suspension, prepared using the same protocol as for transplantation, was plated in 24-well poly-*D*-lysine cover-slipped plate and fixed with 4% PFA. The cells were blocked with 3% serum and 1% bovine serum albumin followed by incubation with the primary antibody overnight at 4 ^{0}C. Then they were washed and incubated with either fluorescent secondary antibody (1:500, Alex flour) or biotinylated IgG secondary antibody (1:200, Vector lab) followed by adding Texas Red Avidin D

stain (1:200, Vector Lab). This process was repeated with a second antibody. Coverslips were mounted using VECTASHIELD® mounting media with DAP (Vector lab) and examined under LEICA DMIRE2 microscope. The primary antibodies were tyrosine hydroxylase (1:400; Abcam); GHSR1a (1:50, Alomone Labs); GOAT (1:200, Phoenix Pharmaceuticals); SOX2 (1:50; Santa Cruz Biotechnology); beta III Tubulin antibody (1:50; Abcam).

Protein lysis and western blot

Frozen tissue samples from striatum, substantia nigra, frontal cortex and hippocampus of 3 adult female SD rats and 7 VM sections from embryos of three E14 Wistar rats were mixed with lysis buffer solution containing anti-protease and anti-phosphatase solutions. Samples were homogenised in tubes containing ceramic beads using Precellys®24 homogenizer followed by centrifugation to remove undissolved impurities. protein concentration in the supernatant was estimated using Bicinchoninic acid assay kit followed by mixing the protein solution with Laemmli lysis buffer and denaturation at 90 for 5 min. The protein samples were loaded in 10% SDS-PAGE running gel and separated in an electrophoresis chamber at 200 V for 1-3 hrs. Then the protein extract was transferred onto nitrocellulose blotting membrane using a semi-dry transfer method. The membrane was blocked with 5% skimmed milk and left with primary antibody overnight at 4 ^{0}C (GHS-R1a 1:200, Santa Cruz Biotechnology; GAPDH 1:15000, Sigma). The membrane was washed and incubated with secondary antibody for 1hr followed by washing and developing the bands using SuperSignal® West Dura kit. After that, the bands were visualised using gel imaging Syngene® G BOX linked to an automatic control software (GeneSys).

Statistical analysis

Statistical analysis was performed using IBM SPSS. The data was normally distributed when tested with Kolmogorov-Smirnova applied with Lilliefors correction. The normally distributed data have homogenous variance when checked with Leven test. Repeated measures analysis ANOVA was performed to analyse behavioural data followed by Bonferroni post hoc test; time was considered as the within subject factor while the groups (lesion; graft+saline; graft+ghrelin 10; graft+ghrelin 50; graft+JMV-2894) were considered as the between subject factor. The histological data was analysed using one-way ANOVA followed with Dunnett's post-hoc test. The specific significance was assumed to 95% (*P<0.05); 99% (**P<0.01) and 99.9% (*** P< 0.001).

Results

Ghrelin receptor GHS-R1a and related enzymes

E14 VM cells variously expressed SOX2, BIII tubulin and TH. GHSR1a co-localised with BIII tubulin and Sox2 demonstrating that it is present in neurons and in immature cells or astrocytes at the time of transplantation. GHSR1a was expressed in most of the VM cells and it was co-expressed with TH indicating that transplanted dopaminergic neurons express the receptor. Similarly, GOAT was co-expressed with either SOX2 or BIII tubulin and TH (Figure 2). Western blot identified the GHSR1a in embryonic VM, and adult SN, striatum, hippocampus and frontal cortex samples (Figure 4). Post mortem double fluorescent immunohistochemistry illustrated the presence of GHSR1a on the TH labelled cells in the grafted striatum demonstrating that the target was present throughout transplantation to graft maturity (Figure 3).

Amphetamine-induced rotation

Lesion-only control rats showed a sustained ipsilateral rotational response after amphetamine administration, while rotations were reduced in all transplanted groups. The data analysis showed a significant difference between the groups ($F_{(4,29)} = 15.4$, $p<0.001$) and significant interaction between group and time ($F_{(4,29)} = 7.8$, $p< 0.001$). Analysis at each time point showed that all transplanted rats had a significant reduction at weeks 4, 6, 8 post grafting compared to lesion control ($p < 0.001$); and there was no difference between the transplanted groups treated with acyl-ghrelin (both doses) or JMV-2894 compared to graft plus saline group (Figure 5).

Motor tasks

There is no significant difference between the groups in motor tasks (max: vibrissae test: $F_{(4,29)} = 1.8$, n.s). In the cylinder test, there was a significant group effect ($F_{(4,29)} =3.2$, $*p<0.027$), however pairwise comparison adjusted with Bonferroni post hoc test showed that there was no significant difference between lesion group and graft plus saline group; and there was no difference between the transplanted groups treated with acyl-ghrelin (both doses) or JMV-2894 compared to graft plus saline group. The only identified difference was between the lesion group and graft plus ghrelin 50 µg group ($p<0.05$), which is a not relevant comparison in this experiment (Figure 5).

Dopaminergic neuron survival in the graft and lesion in the SN:

Grafted TH positive cells were labelled in the striatum and quantified: graft + saline = 891.4 (±398); graft + acyl-ghrelin 10µg = 434 (±164); graft + acyl-ghrelin 50 µg = 375 (± 153); graft + JMV-2894

= 597 (± 226). There was no significant effect of any drug treatment on graft size (one-way ANOVA, p= 0.73, n.s.), which was reflected in the consistent behavioural outcomes (Figure 6). To demonstrate that the behavioural effects were solely due to graft and not due to any recovery of the nigrostriatal pathway the lesion extent of all groups was carefully determined. The percentage of TH positive cell loss in the ipsilateral substantia nigra compared to the intact, contralateral side exceeded 97% in all groups.

Effects of acyl-ghrelin and JMV-2894 on the hippocampal neurogenesis

Consistent with the literature, in the intact side, acyl-ghrelin showed a significant increase in DCX positive cell density in the subgranular zone of the hippocampal dentate gyrus at 10 µg/kg (one-way ANOVA, *p= 0.02) and it caused a tendency to increase at 50 µg/kg compared to saline and lesion controls. In contrast, the animals treated with JMV-2894 showed no difference. On the lesioned/ transplanted side, acyl-ghrelin 10 µg/kg illustrated a tendency to increase the DCX level without significance, while acyl-ghrelin 50 µg/kg and JMV-2894 showed no difference (figure 7).

Discussion

The gut-brain axis has been widely discussed in relation to relevance to neurodegenerative disease. Whilst often pertaining to the gut microbiota, the role of peptides involved in dietary functions have also been the subject of a lot of interest in relation to neuroprotection, as well as a role for calorie restriction (Hu et al., 2006; Maalouf et al., 2009). Gastrointestinal dysfunction is now widely recognised as an early symptom of Parkinson's disease with a-synuclein aggregations identified in the enteric nervous system (Braak et al., 2006). Ghrelin is reduced in people with Parkinson's disease and their nutrient-related regulation of ghrelin also found to be impaired (Song et al., 2017). This study confirms the previous reports showing expression of GHSR1a mRNA in selected brain regions including the SN, hippocampus and cortex (Zigman et al., 2006) and GHSR1a protein and mRNA in the striatum (Kern et al., 2012). For the first time we show the presence of GHSR1a and GOAT, the receptor responsible for the biological function of acyl-ghrelin and the enzyme responsible for converting the ghrelin to its active form (acylation of the third serine residue) respectively, on E14 VM dopaminergic precursors (Gutierrez et al., 2008) immature dopaminergic neurons destined to become the substantia nigra and ventral tegmental area.

Ghrelin has previously been found to be protective of nigrostriatal neurons, exerting its effect through GHSR1a receptors which has been confirmed by either knocking out the receptor (Andrews et al., 2009) or blocking it with D-Lys-3- GHRP-6 (Moon et al., 2009). GHSR1a is expressed in most of the VM cells which is consistent with a previous study reporting a high density of ghrelin receptors in the mature midbrain (Suda et al., 2018b; Zigman et al., 2006). The availability of the basic components for transduction of the ghrelin signal into the transplanted neurons then increases the opportunity for supportive effects. Importantly, GHSR1a is co-expressed with TH in the grafted cells, confirming that receptor expression is maintained for a least 8 weeks after transplantation of dopaminergic foetal cells into a host striatum. However, despite the presence of the machinery, no protective effect was evident. The lack of effect in this study could be down to pharmacokinetic or pharmacodynamic issues; ghrelin has a short half-life, 9-11 min, and was administered as a single daily dose. Nevertheless, this approach was consistent with previous studies although the model differed, the MPTP mouse model being a systemic toxin that targets nigrostriatal neurons (Moon et al., 2009). Importantly, the mechanism through which the nigrostriatal damage occurs in the MPTP model is inhibition of the mitochondrial enzymes in the respiratory chain, a mechanism that is not directly linked with cell death in the early transplantation period. Cells are transplanted into an ectopic site into the striatum and it is reasonable to hypothesise that this dose frequency, even it was protective for the nigral dopaminergic neurons, may not be capable of supporting the survival of dopaminergic neurons in an ectopic environment.

Alternatively, given that Bayliss et al (2016) utilised mice, it could be a species-specific effect that is not mirrored in rats.

JMV-2894 is a long acting GHSR1a agonist which similarly did not show any effect on transplanted cell survival or functional efficacy. The ability of JMV-2894 to cross the blood brain barrier is unconfirmed, however, Moullin and colleagues found that JMV-2894 (compound number 41 in the paper) was not capable of increasing food intake in rats through peripheral administration, although it was able to increase growth hormone levels (Moulin et al., 2007). The use of both compounds was intended to differentiate between a direct central effect or the neurological consequences of a peripheral activation of GSHR1a. To resolve whether there was a functional effect of either compound, we determined whether the ghrelin or ghrelin mimetic altered hippocampal neurogenesis. Previous work indicated that ghrelin has a significant effect in this brain region, increasing the number of DCX labelled neuroblasts in the dentate gyrus following peripheral administration of 10 µg/kg once daily for 14-days in Wistar rats (Kent et al., 2015) and 80 µg/kg once daily for 8-days in Sprague Dawley rats (Li et al., 2013). Our data, using Sprague Dawley rats, confirmed this positive effect of acyl-ghrelin at low dose 10 µg/kg on the hippocampus of both the intact and lesioned/ transplanted hemispheres, while JMV-2894 had no effect. It is worthy of comment that this effect was smaller than previously reported but with a prolonged study in this case we may have missed an earlier peak effect which has now stabilised. Nevertheless, this is clear evidence that adequate dosing of ghrelin over 8 weeks was able to produce a biological effect, whilst JMV-2894 was unable to. This adds greater weight to the evidence that JMV-2894 fails to cross the blood barrier and that the effects of ghrelin are central mediated, and not a consequence of altered peripheral hormones levels.

The unilateral 6-OHDA nigrostriatal lesion, utilised here to mimic part of the pathology of Parkinson's disease, creates an imbalance in striatal dopaminergic drive which can be exploited pharmacologically. Amphetamine induces dopamine release from the intact nigrostriatal pathway, enhancing the motor asymmetry created by the lesion. This imbalance in dopaminergic drive causes an ipsilateral rotational motor response, which is exquisitely sensitive to changes in striatal dopamine levels produced by the transplantation of VM cells (Hefti et al., 1980; Torres and Dunnett, 2007). Importantly for studies of graft survival, the outcome of the rotational test can actually exceed 100% and larger grafts can lead to an overall reversal of the behavioural response (Lane et al., 2006). The reduction in the amphetamine-induced rotational response observed here indicates that the transplanted allogeneic VM cells were functionally capable of releasing a modest amount of dopamine in all transplanted groups. However, these grafts were inadequate to significantly improve motor performances in other tests of motor function. This is attributed due to the graft size being relatively small. Previous studies

illustrated that there may be a threshold level required of the graft size needed to improve motor deficits evident in hand tests, in contrast to the pharmacologically driven amphetamine test in which improvement can be seen with a very small number of cells (Brundin et al., 1988). Nevertheless, this small graft is optimal for the histological analysis to evaluate whether acyl-ghrelin and JMV-2894 have the potential to increase cell survival as indicated by graft size. Due to a higher than anticipated variability in the number of surviving grafted cells, the study was under powered with regards TH expression, although the most sensitive behavioural readout (amphetamine-induced rotations), was more than adequately powered to detect group effects at 0.99. Despite the presence of the appropriate receptor signalling machinery on the cells, neither behavioural nor histological analyses evidenced any effect of either acyl-ghrelin or the agonist JMV-2894 on graft viability, maturation or function. These observations lead to the conclusion that despite published evidence of neuroprotective and neurogenic effects, peripheral administration of ghrelin and JMV-2894 at the doses used have neither a supportive nor detrimental effect on transplanted dopaminergic cells.

Despite the accumulating evidence that supports the protective effect of ghrelin on dopaminergic neurons in different models (Bayliss et al., 2016) of PD, this study clarified that peripheral administration of ghrelin and JMV-2894 were not able to support the survival or efficacy of VM dopaminergic neurons transplanted into the striatum. This was the case despite the presence of both the receptors and GOAT enzymes in the grafted cells maintained through maturation. It remains to be determined whether targeting the receptor with longer acting molecules may yield neuroprotective efficacy in this model.

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Figure Legends

Figure 1 The experimental timeline indicating the motor assessment intervals and dosing schedule.

Figure 2 GHSR1a and GOAT expression on VM cells: Immunocytochemistry on E14 VM cells identified expression of GHSR (red, Texa Red Avidin D) co-localised with TH, sox2 and BIII tubulin labelled cells (green, Alex flour 488). Similarly, Ghrelin O Acyl transferase enzyme GOAT (red, Alex flour 594) expressed on TH, SOX2 and BIII tubulin labelled cells (green, Alex flour 488). Arrows indicate co-localised targets.

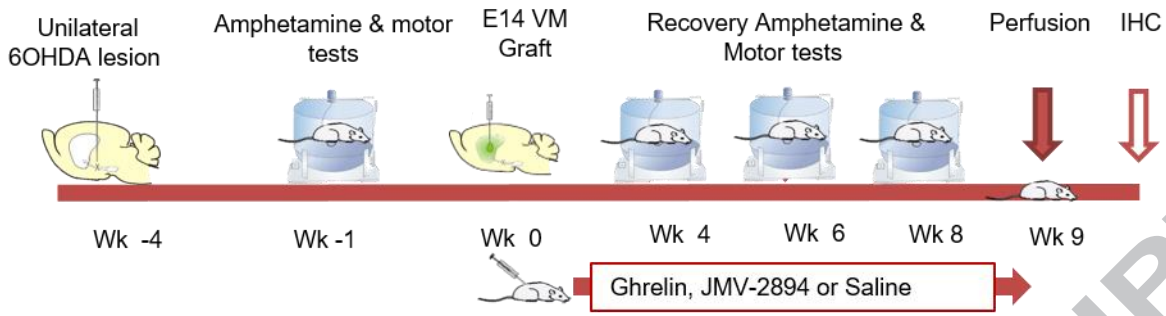
Figure 3 GHSR1a expression in the graft: illustrating co-localisation of TH (green, Alex flour 488) and GHSR1a (red, streptavidin cy3) on the graft. Arrows indicate co-localised targets.

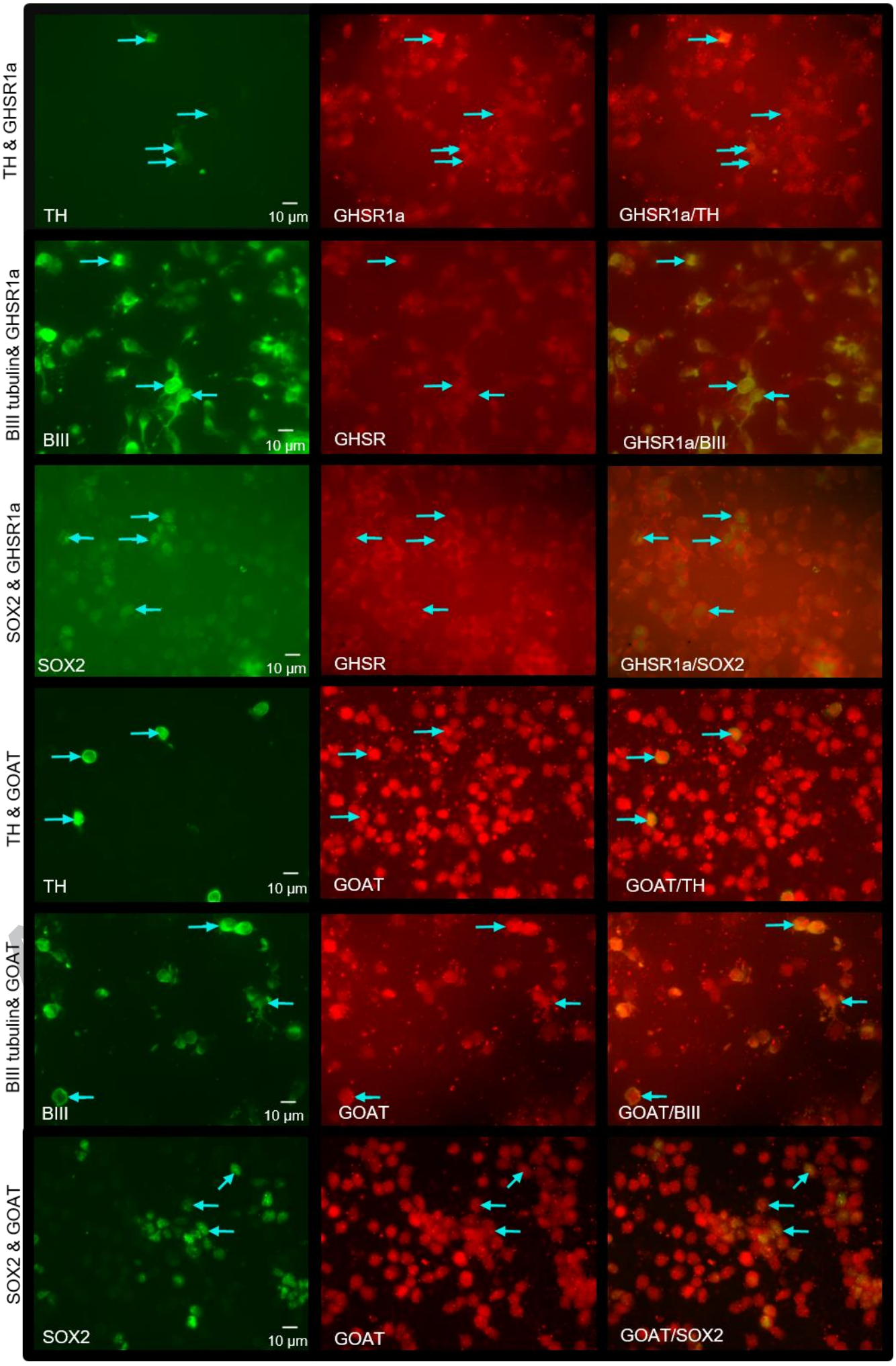
Figure 4 Western Blot analysis: WB showed detection of GHSR1a protein (size 41Kda) in tissue samples dissected VM sections and from the following brain regions of adults female SD rats: SN = substantia nigra ; Hip = hippocampus; Str = striatum; and Ctx = frontal cortex. The GAPDH protein was used as a house-keeping and detected at size 37 kDa.

Figure 5: Motor and behavioural outcomes following transplantation of dopaminergic primary neurons in the presence of saline, Ghrelin 10ug or 50ug or JMV-2894. (A) Amphetamine induced rotation: all grafts ameliorated ipsilateral rotations significantly since week 4 of transplantation (***P< 0.001) comparing to lesion only control. (B) cylinder test, (C) vibrissae test and (D) stepping test illustrated on effect of the graft plus saline to improve rats' performances compared to lesion group whilst neither dose of ghrelin nor JMV-2894 had additional effects compared to the graft plus saline control group.

Figure 6 TH cells counting in the graft and SN: (A) mean number of TH positive cells in the graft showing no effect of treatments with either ghrelin or JMV 2894 comparing to saline control group (one-way ANOVA analysis). (B) The percentage of the degenerated TH positive cells in the lesioned side of the substantia nigra compared to the intact side was more than 97% in all groups.

Figure 7: effect of treatments on hippocampal neurogenesis in the lesion and intact hemisphere: (A) DCX labelled cells density in the DG of the lesion/ transplant side: the lower dose-ghrelin group showed a tendency to increase DCX density without significant difference. (B) DCX labelled cells density in the DG of the intact hemisphere: only the lower dose of ghrelin significantly increased DCX density compared to the control groups (*p< 0.05)




TH & GHSR1a
TH
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BIII
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SOX2
10 μm
GHSR
GHSR1a/SOX2
TH & GOAT
TH
10 μm
GOAT
GOAT/TH
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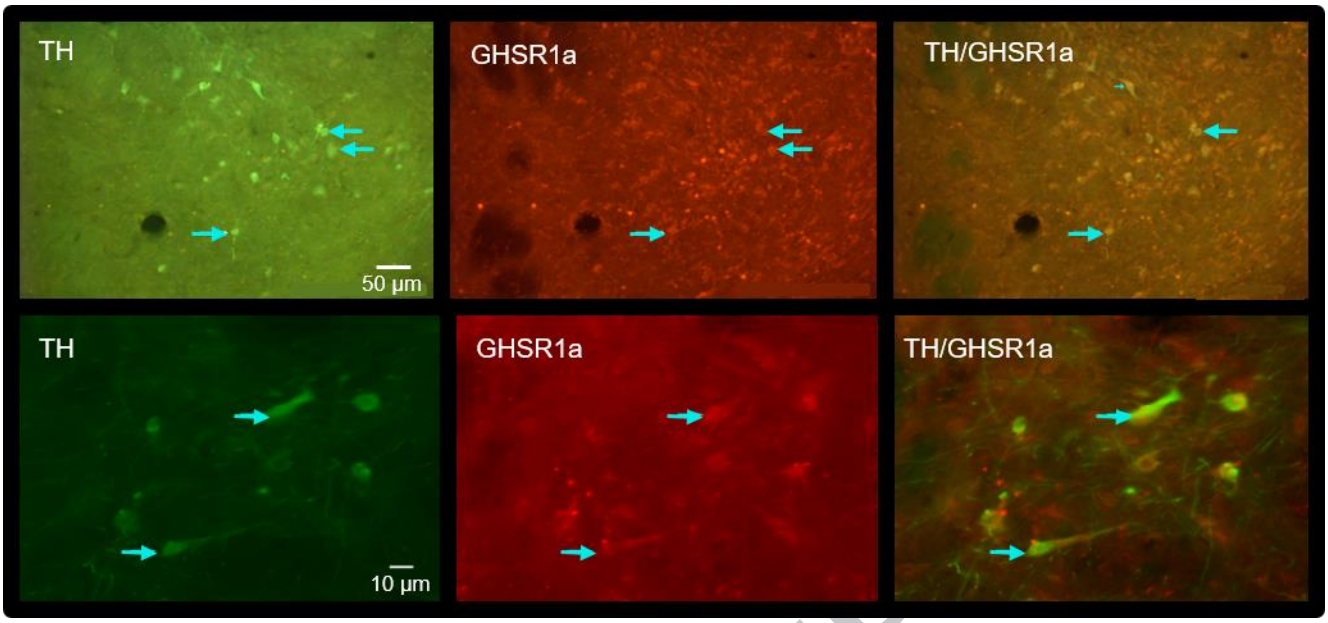

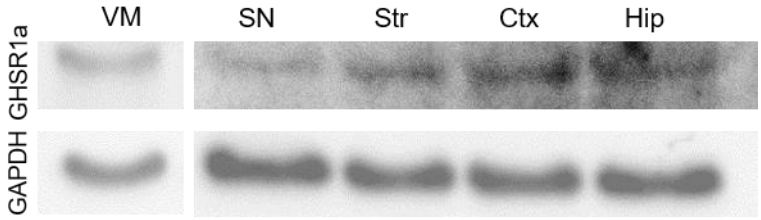

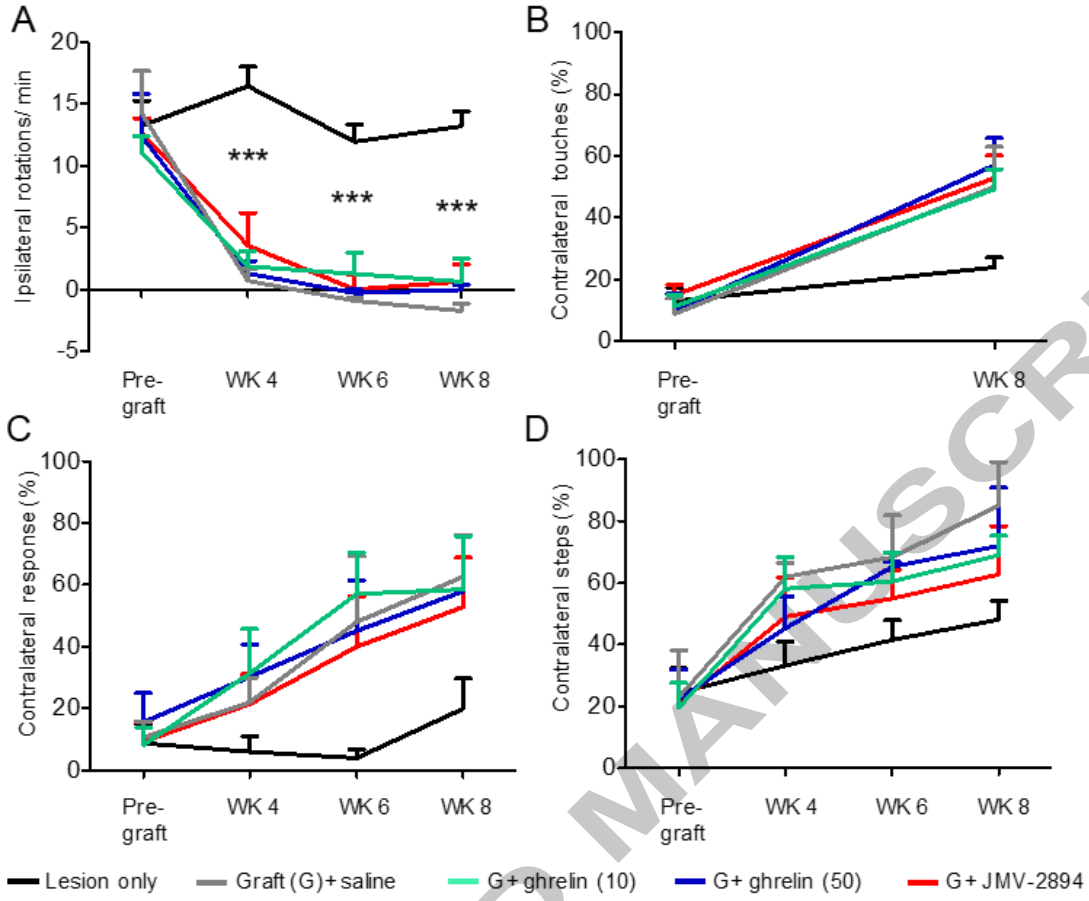

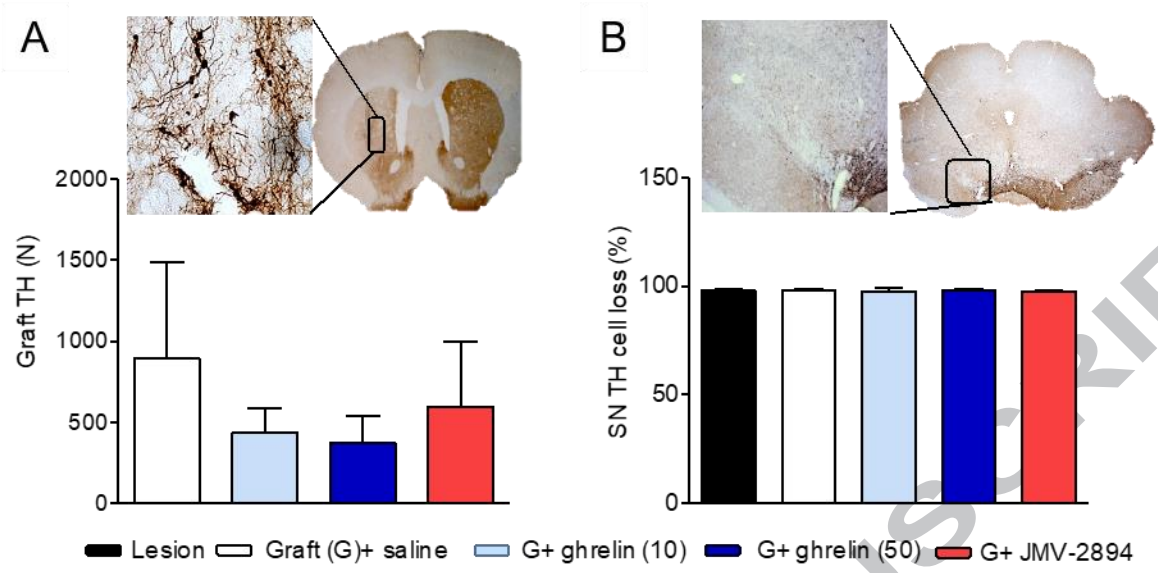

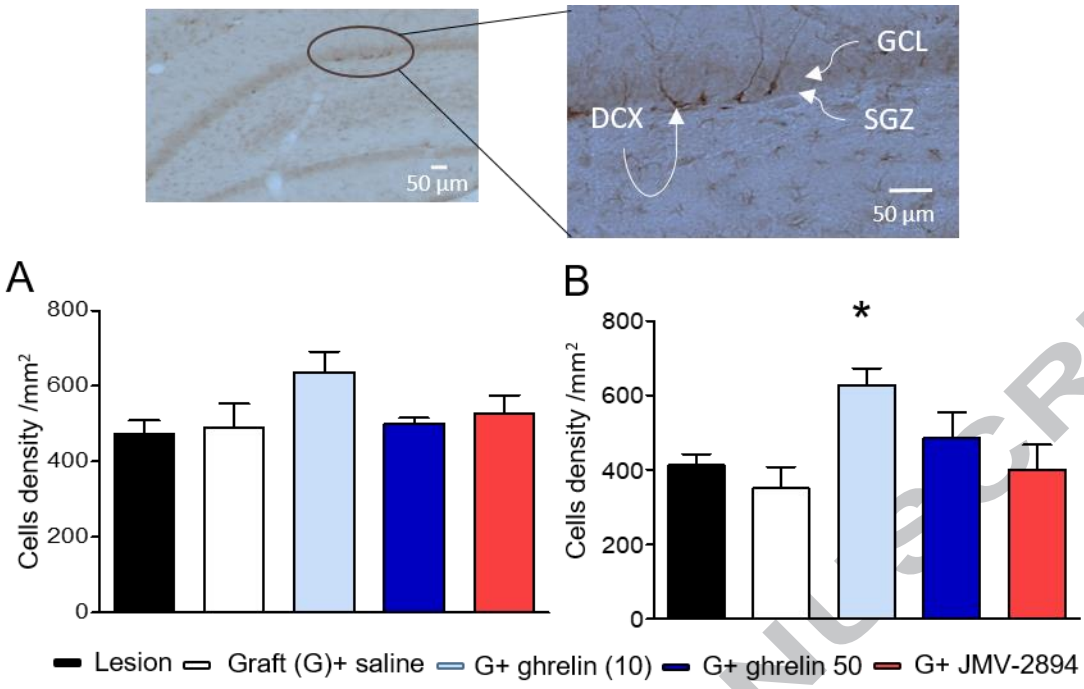

1. GHSR1a was detected on E14 ventral mesencephalic cells
2. Expression persists in an intrastriatal graft into 6-OHDA rats.
3. Ghrelin and JMV-2894 had no effect on graft survival or function.
4. Ghrelin-induced hippocampal neurogenesis confirmed efficacy.