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Septal glucagon-like peptide 1 receptor expression determines suppression of cocaine-induced behaviour

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Running title: Septal GLP-1Rs mediate psychomotor effects of cocaine

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Abstract

Glucagon-like peptide 1 (GLP-1) and its receptor GLP-1R are a key component of the satiety signalling system and long-acting GLP-1 analogues have been approved for the treatment of type-2 diabetes mellitus. Previous reports demonstrate that GLP-1 regulates glucose homeostasis alongside the rewarding effects of food. Both palatable food and illicit drugs activate brain reward circuitries, and pharmacological studies suggest that central nervous system GLP-1 signalling holds potential for the treatment of addiction. However, the role of endogenous GLP-1 in the attenuation of reward-oriented behaviour, and the essential domains of the mesolimbic system mediating these beneficial effects, are largely unknown. We hypothesized that the central regions of highest *Glp-1r* gene activity are essential in mediating responses to drugs of abuse. Here, we show that *Glp-1r*-deficient (*Glp-1r*$^{-/-}$) mice have greatly augmented cocaine-induced locomotor responses and enhanced conditional place preference compared with wild-type (*Glp-1r*$^{+/+}$) controls. Employing mRNA *in situ* hybridization we located peak *Glp-1r* mRNA expression in GABAergic neurons of the dorsal lateral septum, an anatomical site with a crucial function in reward perception. Whole-cell patch-clamp recordings of dorsal lateral septum neurons revealed that genetic *Glp-1r* ablation leads to increased excitability of these cells. Viral vector-mediated *Glp-1r* gene delivery to the dorsal lateral septum of *Glp-1r*$^{-/-}$ animals reduced cocaine-induced locomotion and conditional place preference to wildtype levels. This site-specific genetic complementation did not affect the anxiogenic phenotype observed in *Glp-1r*$^{-/-}$ controls. These data reveal a novel role of GLP-1R in dorsal lateral septum function driving behavioural responses to cocaine.

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Introduction

The shift from controlled to compulsive drug use leading to addiction is poorly understood but seems to involve pathways originally evolved for natural rewards like food (Volkow and Wise, 2005). Recent preclinical studies suggested that GLP-1 modulates the rewarding effects of both food (Alhadeff *et al*, 2012; Dickson *et al*, 2012; Dossat *et al*, 2011) and addictive drugs (Egecioglu *et al*, 2013a, b; Egecioglu *et al*, 2013c; Erreger *et al*, 2012; Graham *et al*, 2013). This was supported by the observation that pre-treatment with the synthetic GLP-1R agonist Exendin-4 (Ex4) attenuates the behavioural effects of drugs of abuse including ethanol (Egecioglu *et al*, 2013c; Shirazi *et al*, 2013), nicotine (Egecioglu *et al*, 2013a), amphetamine and cocaine (Egecioglu *et al*, 2013a, b; Erreger *et al*, 2012; Graham *et al*, 2013). Therefore, GLP-1R agonists have been proposed as treatment options for drug addiction. Most studies employed the systemic administration of GLP-1R ligands, obviating the dissection of peripheral versus central GLP-1R effects. Interestingly, GLP-1R activation in the ventral tegmental area (VTA) mediates the rewarding effects of alcohol (Shirazi *et al*, 2013) and palatable food (Mietlicki-Baase *et al*, 2013). In addition, systemic Ex4 decreases the release of dopamine in the nucleus accumbens (NAc) (Egecioglu *et al*, 2013a, b). It remains to be determined whether these effects are due to a direct activation of GLP-1R in the NAc and VTA, or rather a result of modulations on a circuit level.

In the central nervous system (CNS), GLP-1 is produced by neurons in the nucleus of the solitary tract (NTS) that project to various regions, including sites that regulate stress responses, as well as reward and motivation (Llewellyn-Smith *et al*, 2011; Rinaman, 2010). The immunohistochemical detection of GLP-1R expression in the brain is unknown as specific anti-GLP-1R antibodies are not available (Pyke and Knudsen, 2013), yet ligand binding assays suggest the presence of the receptor in

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the amygdala and the mesolimbic system, including the VTA, NAc and lateral septum (LS), providing an anatomical correlate for the reported beneficial effects of pharmacological GLP-1R stimulation (Göke, 1995).

The LS is highly interconnected with the mesolimbic system (Olds and Milner, 1954; Sheehan, 2004) primarily through γ-aminobutyric acid (GABA)ergic projections (Gallagher *et al*, 1995; Risold and Swanson, 1997a). LS neurons form a strong collateral network (Gallagher *et al*, 1995; Sheehan, 2004), which causes not only recurrent inhibition within the LS, but also tightly controls the activity of extra-septal target areas (Sheehan, 2004). The LS regulates responses to natural rewards (Noda *et al*, 1997) and is involved in stress-induced relapse to drug abuse (Highfield *et al*, 2000). Hence, the LS is a hub that integrates information arriving from central key regulators and mediates appropriate behavioural responses (Sheehan, 2004). A recent study identified a novel circuit, linking context and reward, which relies on neurons of the dorsal LS (dLS) (Luo *et al*, 2011). This circuit starts with hippocampal glutamatergic projections, which stimulate GABAergic neurons in the dLS. These dLS neurons project to the VTA, leading to disinhibition of dopaminergic neurons within the VTA, and a subsequent increase of dopamine levels in the NAc (Luo *et al*, 2011; Mazei-Robison *et al*, 2011).

The identification of mechanisms that regulate LS activity is therefore important for understanding the circuits of the mesolimbic reward system as well as the neurobiology of drug-induced neuroplasticity and drug reinforcement. Here, we used a genetic mouse model with a targeted null-mutation of the *Glp-1r* locus (Scrocchi *et al*, 1996) enabling the determination of behavioural responses to cocaine administration that were dependent upon GLP-1-mediated LS-neuromodulation.

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Materials and Methods

Animals. Experiments were carried out in accordance with the UNSW Australia Animal Care and Ethics Committee. Heterozygous *Glp-1r*$^{+/-}$ breeding pairs were used to produce age matched male *Glp-1r*$^{-/-}$ mice (Scrocchi *et al*, 1996) on C57BL/6J background and *Glp-1r*$^{+/+}$ littermates. PCR-genotyping was performed as described previously (Kim *et al*, 2013). Animals were individually housed in a temperature- and humidity-controlled room with a 12h light/dark cycle and had access to food and water *ad libitum*.

Adeno-associated virus (AAV) constructs. The rat *Glp-1r* open reading frame was cloned upstream and in-frame with the coding region of the enhanced green fluorescent protein (GFP) reporter in an AAV expression cassette containing the 1.1kb chicken β-actin promoter (CBA), the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE), and the bovine growth hormone polyadenylation sequence (bGHpA) flanked by AAV2 inverted terminal repeats (pAAV-GLP-1R-GFP). The same backbone carrying no *Glp-1r* cDNA was used as a control (pAAV-GFP). In addition, a non-tagged GLP-1R construct (During *et al*, 2003) was used as a control for experiments in human embryonic kidney (HEK293) cells.

Immunoblotting. HEK293 cells were transfected with pAAV-GLP-1R-GFP, pAAV-GLP-1R, or pAAV-GFP, using the standard calcium phosphate ($CaPO_4$) precipitation method. 48h after transfection the cells were exposed to Ex4 (10 nM) for 0-50 min before harvesting in solubilization buffer (30 mM Tris-Cl, pH 7.4, and 5 mM $MgCl_2$ containing 4 mg of CHAPS (Sigma) and 20% glycerol). 10 µg of total protein were resolved by 12% SDS-PAGE and electro-blotted onto nitrocellulose membrane. The membrane was incubated with primary antibodies against phosphorylated

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extracellular signal-regulated kinases (mouse anti-pERK1/2; 1:2500; Cell Signaling, Danvers, USA) and the GFP-tag (rabbit anti-GFP; 1:2000 (von Jonquieres *et al*, 2013)). The membrane was re-probed using a rabbit anti-ERK1/2 antibody (1:2500; Cell Signaling). Secondary antibodies were horseradish peroxidase-conjugated as required (1:5000; Dianova, Hamburg, Germany). Immunoreactivity was detected using the enhanced chemiluminescence system (BioRad, Gladesville, NSW, Australia) and digitalized (GelDoc, BioRad, Gladesville, NSW, Australia).

AAV vector generation and administration. Packaging of neurotropic AAVrh10 vectors was performed as described previously (Klugmann *et al*, 2005). Briefly, following triple transfection of HEK293 cells, AAV vectors were purified using iodixanol (OptiPrep™, Sigma-Aldrich) gradient ultracentrifugation (Zolotukhin *et al*, 2002). Titres were determined by quantitative real-time PCR of vector genomes using primers for WPRE. One µl of either AAV-GLP-1R-GFP or AAV-GFP (8×10^{11} vg/ml) was injected bilaterally into the dLS (+0.5 mm AP, ±0.3 mm ML, -2.6 mm DV from bregma). Vectors were delivered to 4 week-old mice at a rate of 150 nl/min using a microprocessor-controlled mini-pump (World Precision Instruments, Sarasota, FA, USA) with 34G bevelled needles (World Precision Instruments) in a stereotaxic frame (Kopf Instruments, Tujunga, CA, USA).

Immunohistochemistry. Brains of all animals were assessed for transgene expression at the end of behavioural testing. Immunostaining of brain sections was performed as described (Klugmann *et al*, 2005). Briefly, mice were transcardially perfused with 10% buffered neutral formalin (Sigma-Aldrich, Taufkirchen, Germany), removed and post-fixed for 2h in the same fixative before cryoprotection in 30% sucrose/PBS. Free-floating cryosections (40 µm) were rinsed with PBS-Triton-X100 (0.2%), blocked

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in immunobuffer (4% horse serum in PBS, pH 7.4, 0.4% Triton-X100), followed by incubation with rabbit anti-GFP (von Jonquieres *et al*, 2013)(1:1000). Sections were then incubated with donkey anti-rabbit Alexa488 antibody (1:1000; Invitrogen, Karlsruhe, Germany) and counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Sections were mounted and coverslipped with Mowiol (Sigma-Aldrich). Fluorescence was visualized using a confocal microscope (Zeiss Z1 AxioExaminer NLO710; Carl Zeiss, North Ryde, NSW, Australia).

In situ hybridization (ISH). ISH was performed as previously described (Marsicano and Lutz, 1999). Both digoxygenin (DIG)-labelled and fluorescein isothiocyanate (FITC)-labelled riboprobes were used as described elsewhere (Yang *et al*, 1999). The cDNAs encoding glutamic acid decarboxylase (GAD65), mouse GLP-1R (GenBank accession number: NM_021332) in the pBlueskript plasmid backbone, including a *Glp-1r* sense probe, were used for *in vitro* transcription. Hybridization was carried out overnight at 55 °C in 100 μl of buffer containing DIG-labelled (300 ng/ml) *Glp-1r* riboprobe for single ISH or, in case of double ISH, additional FITC-labelled *Gad65* riboprobe (300 ng/ml). Sections were counterstained with DAPI or toluidin blue, coverslipped in Mowiol or DPX (Sigma-Aldrich). Fluorescent sections were visualized using a confocal microscope; DIG-labelled sections were visualized using an Olympus BX51 microscope. Semi-quantitative analysis, in the coronal plane cut through bregma, was performed by detemining the number of *Glp-1r* mRNA expressing cells among the total population of dLS neurons identified by soma size, or *Gad65* mRNA expressing neurons. Samples were collected from three *Glp-1r*$^{-/-}$ and *Glp-1r*$^{+/+}$ animals (three sections per animal, spaced 80 μm). Digitalized images were processed with Adobe Photoshop CS5 or ZEN lite (Zeiss) where appropriate.

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Electrophysiology. Coronal brain slices (300 μm) were prepared from mice (4–8 weeks) using a VT1200 vibratome (Leica) according to standard techniques (Power and Sah, 2007). Briefly, mice were anesthetized with isoflurane (5%) and decapitated, the brains removed and sectioned in oxygenated (95% O_2, 5% CO_2) ice-cold, modified artificial cerebrospinal fluid (ACSF) containing the following (in mM): 125 NaCl, 3 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 6 $MgCl_2$, 1 $CaCl_2$, 10 glucose. Slices were incubated at 34 °C for 30 min and thereafter maintained at room temperature in ACSF solution (in mM): 125 NaCl, 3 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 1 $MgCl_2$, 2 $CaCl_2$, 25 glucose (equilibrated with 95% O_2, 5% CO_2). Whole-cell patch-clamp recordings were made from visually identified neurons (Zeiss Axio Examiner D1) in slices continuously superfused with ACSF (1–2 ml/min) heated to 30 ± 1 °C using an in-line heater (TC-324B Warner Instrument Corporation). Patch pipettes (2-4 MΩ) were filled with internal solution (in mM): 135 $KMeSO_4$, 7 NaCl, 10 HEPES, 2 Mg_2ATP, 0.3 Na_3GTP, pH7.3 with KOH (osmolarity, 280-300 mOsm/kg). Electrophysiological recordings utilized an Axopatch 200B amplifier (Molecular Devices), filtered at 2-5 kHz, digitized at 20 kHz with a Digidata1440 (Molecular Devices) interface, controlled using PClamp 10.2 software (Molecular Devices). Current was injected to adjust the membrane potential to -65mV, and the voltage response to somatic current injections (600 ms, -50 to +150 pA, 10 pA increments) was used to measure the neuron's AP firing pattern. Action potential latency was calculated from the first action potential evoked at the threshold current injection. Neurons with resting potentials > -55mV were excluded from the dataset. Voltage-clamp was employed to dissect dLS membrane conductances, in particular A-type K^+-currents (Supplementary data).

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Behavioural testing. All animals were adult (2-4 months) and individually housed two weeks prior to behavioural testing to prevent abnormal behaviour caused by fighting or suppression by the alpha male. Experience in our lab shows that reward-related behavior does not change in C57bl6/J mice between 2-6 months. All animals were subjected to the following battery of tests. The elevated plus maze (EPM) was employed to assess inherent anxiety-related behaviour. The maze consisted of two opposite open arms and two opposite enclosed arms, interconnected by a central platform (centre 6x6 cm^2; open arms, 35x6 cm^2; closed arms, 35x6x15 cm^3; elevation 1 m; illumination 40 lux). Mice were placed on the maze, facing a closed arm and were allowed to freely explore the EPM for 5 min. Mouse behaviour was analyzed using ANY-maze™ tracking software (Stoelting, Illinois, USA). The ratio of time spent in open arms was used to estimate open arm aversion (reflecting anxiety). The same cohorts of animals were subjected to two non-contingent models of substance abuse (Kalivas, 2009). Briefly, clear Plexiglas boxes (41x41x40 cm; Photobeam Activity System: MedAssociates Inc, St Albans, VT, USA) were used as open field boxes for recording the locomotor sensitization to cocaine (30 min sessions per test day). After an initial day with saline injections (baseline activity), animals received cocaine (10 mg/kg, IP) for 5 consecutive days (Day 1-5; with Day 5 representing the development of sensitization). After 7 consecutive drug- and test-free days, all mice received a priming injection of cocaine (Day 12; representing the expression of sensitization). The last day of sensitization was followed by 14 test-free days before the same animals were subjected to the conditioned place preference (CPP) test. CPP was evaluated using a modified unbiased protocol as described previously (Bilbao *et al*, 2008). Briefly, CPP was performed in a custom-made box with two equally sized compartments (25x25x40 cm) interconnected by a rectangular corridor. The compartments were differentiated by the pattern of the walls and texture of the

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floor. Mice received one session of preconditioning (15 min), followed by four days of conditioning. During the conditioning phase, mice were treated with eight alternating injections of saline (morning) and cocaine (10 mg/kg; IP; afternoon) and then confined to the corresponding compartment immediately after the injection for 30 min. For the post-conditioning test (performed at noon), mice were allowed to explore the apparatus without any treatment for 15 min. The CPP score represents the difference between the time spent in the cocaine compartment before and following conditioning.

Data analysis and statistics. Current Clamp data were analysed off-line using custom routines written in LabVIEW (National Instruments, Austin, TX, USA). Data are presented as means ± S.E.M. and were analyzed as appropriate using unpaired, two-tailed t-test, or two-way repeated measures ANOVA followed by the Bonferroni posthoc test to evaluate statistical significance. In all cases $P < 0.05$ was considered statistically significant.

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Results

Abundant expression of endogenous *Glp-1r* in the LS.

Glp-1r expressing cells were found in the amygdala, as well as the hypothalamus (Figure 1A). In order to gain an overview of *Glp-1r* gene activity, we utilized expression data publicly available from the Allen Brain Atlas database (Lein *et al*, 2007). High density *Glp-1r* mRNA labeling was detected in the pontine grey but also in the LS (Figure 1B). In contrast, only few or no Glp-1R positive cells were found in the NAc (Figure $1B_1$) or VTA (Figure $1B_2$). Given that LS neurons project to both areas, we further elucidated *Glp-1r* mRNA expression in the LS. Abundant *Glp-1r* expression levels in the LS were corroborated assessing an independent Allen Brain Atlas data set (Figure 1C). We then performed ISH to identify *Glp-1r* expression in the LS in more detail. Hybridization with the sense riboprobe did not result in signal detection confirming absence of non-specific signals (Figure 1D). The antisense riboprobe confirmed strong *Glp-1r* mRNA expression in the dLS as shown in a representative image (Figure 1E). Semi-quantitation of these results revealed that over 95% of dLS neurons express *Glp-1r* mRNA. To identify the phenotype of *Glp-1r*-positive dLS neurons we performed double fluorescent ISH using probes for *Glp-1r* and *Gad65*, the enzyme responsible for GABA synthesis. Virtually all *Glp-1r*-positive cells expressed *Gad65*, indicating that these cells are inhibitory GABAergic neurons (Figure 1F–I). No signal was detected with the antisense probe using brain sections from GLP-1R-deficient mice (not shown).

Ablation of GLP-1R signalling alters intrinsic excitability of septal neurons.

Our anatomical data suggested that septal GLP-1Rs may play an important role in the mesolimbic reward system. We hypothesized that this is caused by GLP-1R-dependent changes in dLS neuronal excitability and therefore analyzed the firing

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properties of *Glp-1r*$^{+/+}$ and *Glp-1r*$^{-/-}$ neurons employing brain slice whole-cell patch-clamp. dLS neurons from *Glp-1r*$^{+/+}$ and *Glp-1r*$^{-/-}$ did not differ in their passive membrane properties (resting potential, input resistance, membrane time constant) or their AP waveform (threshold, amplitude, width), their fast AHP amplitude, or the minimum current required to elicit an AP (Table S1). However, there were differences in the neuronal firing properties. In response to a threshold current injection, the onset of the AP was delayed in *Glp-1r*$^{-/-}$ neurons compared with *Glp-1r*$^{+/+}$ controls (Figure 2A). Increasing the injected current revealed that septal *Glp-1r*$^{-/-}$ neurons were more excitable, firing more APs than *Glp-1r*$^{+/+}$ neurons to twice threshold current injections (Figure 2D) and to current injections >120 pA (Figure 2E, F). The AP onset difference suggested that fast activating voltage-dependent K^+-currents might be altered in *Glp-1r*$^{-/-}$ neurons. Dissecting these currents with voltage-clamp protocols in combination with the pharmacological blockage isolated prominent A-type K^+-currents, that were retained with *Glp-1r* deletion (Figure S1).

GLP-1R in the dLS controls cocaine-induced behaviours.

Our anatomical and electrophysiological data indicated that GLP-1R contributes to dLS function and therefore to behavioural outputs modulated by the dLS (Sheehan, 2004). We hypothesized that assessments of anxiety-related and drug-induced behaviours would reveal deficits in *Glp-1r*$^{-/-}$ mice that were tied to the dLS and hence could be rescued by genetic complementation of GLP-1R in the dLS.

To achieve precise anatomical accuracy for expression of transgenic GLP-1R *in vivo* we employed the AAV platform to induce robust long-term transgene expression following intracranial delivery (Klugmann *et al*, 2005). The AAV expression construct included a GFP-epitope tag at the GLP-1R C-terminus for immuno-detection of the recombinant receptor (Figure 3A). Biological functionality of

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the tagged receptor was confirmed in heterologous HEK293 cells by monitoring the activation of ERK1/2, a known downstream event of GLP-1R signaling (During *et al*, 2003). Levels of phosphorylated ERK1/2 peaked at 10min and declined after 20min following administration of Ex4 (Figure 3B). A doublet at 80kD and 100kD detected by immunoblot (Figure 3B), indicated post-translational modifications. Immunocytochemical detection of recombinant GLP-1R revealed cell surface localization (Figure 3C). These data suggest that the presence of the GFP-tag does not interfere with receptor signalling, posttranslational processing or trafficking.

We then packaged AAV-GLP-1R-GFP or AAV-GFP for bilateral delivery into the mouse dLS. Immunohistochemical assessment of transduction was performed three weeks following vector delivery, when AAV-mediated transgene expression is known to reach maximum and stable levels (Klugmann *et al*, 2005), to confirm accurate anatomical targeting; the subcellular expression patterns matched our observations *in vitro* (Figure 3C). As anatomical confinement of the rescue experiment is essential, we investigated the possibility of off-targeting in the lateral ventricles. Transduced ependymal cells, glia lining the walls of the ventricles, are an inevitable consequence of intraventricular AAV delivery. We critically examined all 15 AAV-animals (n=9 for AAV-GLP-1R; n=6 for AAV-GFP) for this feature and observed GFP-positive ependymal cells in only one case, an AAV-GFP brain, depicted in Figure 3C.

The vector spread extended 2mm in the rostro-caudal dimension (Figure S2) and is illustrated in Figure 3D. AAVrh10 can be transported retrogradely along axonal projections (Cearley and Wolfe, 2007); except a small number of transduced CA3 pyramidal neurons in the dorsal hippocampus (not shown), we did not detect GFP-positive cell bodies in extra-septal regions.

For behavioural assessment we tested AAV-GFP-treated and *naïve Glp-1r*$^{-/-}$ or

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Glp-1r$^{+/+}$ mice. Data of the corresponding AAV-GFP and naïve mice were pooled since they were not statistically different (not shown). A separate cohort entailed *Glp-1r*$^{-/-}$ mice bilaterally injected with AAV-GLP-1R-GFP into the dLS. We employed the EPM test to determine levels of inherent anxiety. *Glp-1r*$^{-/-}$ mice and *Glp-1r*$^{-/-}$ mice injected with AAV-GLP-1R-GFP spent less time in the centre of the maze, showed fewer open arm entries, and spent less time in the open arms compared with *Glp-1r*$^{+/+}$ controls (Figure 4A). All groups displayed comparable locomotor activity (Figure S3A). These results suggest that the loss of GLP-1R in brain regions outside the dLS mediate anxiety-related behaviour.

The same cohorts were then subjected to the locomotor sensitization test as described (see methods). *Glp-1r*$^{-/-}$ mice, but not *Glp-1r*$^{-/-}$ mice injected with AAV-GLP-1R-GFP, showed an augmented acute response to cocaine (Figure 4B; Day 1). Increased locomotor activity was detected in *Glp-1r*$^{+/+}$ controls and *Glp-1r*$^{-/-}$ mice between the Day 1 and Day 5, indicating intact development of sensitization. While development was absent in *Glp-1r*$^{-/-}$ mice injected with AAV-GLP-1R-GFP, this group was not statistically different to *Glp-1r*$^{+/+}$ controls. Following a drug-free period of 7 days, the animals received a cocaine challenge and *Glp-1r*$^{+/+}$ controls and *Glp-1r*$^{-/-}$ mice, but not *Glp-1r*$^{-/-}$ mice injected with AAV-GLP-1R-GFP, showed expression of sensitization (Day 12). Baseline locomotion in the open field box was not different among groups (Figure S3B). All groups showed a similar magnitude of change upon comparison of the difference between Day 5 and Day 1 or between Day 12 and Day 1 (Figure S4A). Therefore the sensitization process per se was not enhanced in *Glp-1r*$^{-/-}$ mice compared with *Glp-1r*$^{+/+}$ controls probably because of the augmented initial sensitivity to cocaine and a ceiling effect in *Glp-1r*$^{-/-}$ mice. Sensitization as well as initial responsiveness to psychomotor stimulants can be exhibited by stereotypy, which is usually associated with a drop in locomotion. However, no alterations

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between groups were observed comparing stereotypic time over the course of the experiment (Figure S4B).

The rewarding effects of cocaine were investigated in the conditioned place preference (CPP) paradigm. *Glp-1r*$^{-/-}$ mice exhibited enhanced CPP compared with *Glp-1r*$^{+/+}$ controls following conditioning using cocaine doses of 10 mg/kg (Figure 4C) or 15 mg/kg (Figure S5) suggesting augmented cocaine-seeking behaviour. This response was restored in *Glp-1r*$^{-/-}$ mice treated with AAV-GLP-1R-GFP (Figure 4C). To rule out that the observed CPP of *Glp-1r*$^{-/-}$ mice is caused by an enhanced conditioned locomotor effect (Huston *et al*, 2013) we analyzed the locomotor activity during CPP expression and detected no difference between groups (Figure S6). *Glp-1r* mRNA expression was increased by 4-fold in the dLS of AAV-GLP-1R-GFP treated *Glp-1r*$^{-/-}$ mutants compared with AAV-GFP treated *Glp-1r*$^{+/+}$ mice using qRT-PCR analysis of brain slice punches (Figure S7). While the corresponding GLP-1R protein quantities could not be determined, it was shown previously that even substantial AAV-mediated transgene expression at mRNA levels translates to physiological protein amounts (Blume *et al*, 2013).

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Discussion

The present study provides clear evidence that GLP-1R positive dLS GABAergic neurons are critical for the mediation of drug-induced behaviour. Several reports have shown that feeding-related peptides, such as leptin or ghrelin, influence the mesolimbic system (Hommel *et al*, 2006; Skibicka *et al*, 2011). The role of GLP-1 and its analogues in this context has only recently been investigated by pharmacological studies supporting the concept that GLP-1R signalling alters behaviours induced by drugs of abuse (Egecioglu *et al*, 2013a, b; Egecioglu *et al*, 2013c; Erreger *et al*, 2012; Graham *et al*, 2013; Shirazi *et al*, 2013). The preclinical potential of GLP-1 based compounds for the treatment of dependence has been broadly appreciated (Skibicka, 2013), and the translational relevance of this research is emerging (van Bloemendaal *et al*, 2014).

Acute injections of GLP-1 or Ex4 into the VTA or NAc reduce reward-driven behaviour, an effect that was attributed to GLP-1R activation in these brain areas (Dickson *et al*, 2012; Dossat *et al*, 2011). The direct action of GLP-1 appears conceivable in light of classical ligand binding data reporting the presence of GLP-1R in these areas (Göke, 1995). Moreover, GLP-1 producing NTS neurons project into key areas of the mesolimbic system including the VTA and NAc (Merchenthaler, 1999; Rinaman, 2010). However, only mRNA ISH data inform specifically on the neuroanatomical sites of *Glp-1r* producing neurons; while sparse *Glp-1r* mRNA levels were reported in the aforementioned parts of the mesolimbic system (Merchenthaler, 1999), the current study identified most abundant central *Glp-1r* mRNA expression in the dLS (Figure 1B,C) the functional relevance of which has not been appreciated yet. Broadly, the LS is connected with brain regions implicated in regulating mood and reward (Risold and Swanson, 1997b) and strongly influences motivated behaviours (Olds *et al*, 1954; Prado-Alcala *et al*, 1984).

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Here, we utilized genetic GLP-1R models on the C57BL/6J background and showed that the complete ablation of GLP-1R caused an anxiogenic phenotype that was not restored by dLS directed AAV-*Glp-1r* gene delivery, suggesting that extra septal GLP-1Rs (i.e. amygdala) mediate anxiety-related behaviour. Inconclusive results have previously been reported for EPM test performance of GLP-1R-deficient mice on a CD1 background (MacLusky, 2000). Considering strain differences, that are known to play a critical role in mediating behavioural responses, CD1 mice showed an decrease in anxiety-related behaviour compared to the C57BL/6J background (Michalikova *et al*, 2010).

Glp-1r$^{-/-}$ mice showed augmented cocaine-induced acute locomotor response and increased CPP, indicating enhanced reinforcement and stimulatory effects of cocaine in these animals. This phenotype was corrected by the genetic rescue confined to the dLS indicating that GLP-1R in neurons from that brain region is essential for motivated behaviour.

The peripheral delivery of Ex4 in mice and rats reduces cocaine and amphetamine-induced hyperlocomotion (Egecioglu *et al*, 2013b; Erreger *et al*, 2012; Graham *et al*, 2013); yet these pharmacological studies utilized protocols limited to monitor acute psychostimulant responses. In contrast, we employed an established sensitization paradigm testing both acute and chronic responses (Bilbao *et al*, 2008), revealing that complete GLP-1R ablation enhanced cocaine-induced locomotor activity throughout the test. As such our findings are in line with the previously reported ability of Ex4 to block the expression of nicotine-induced locomotor sensitization (Egecioglu *et al*, 2013a). The persistent nature of the sensitized motor response has been proposed to reflect neurochemical alteration similar to the enduring changes associated with addiction, indicating potentially shared neuronal substrates (Kalivas, 2009; Robinson and Berridge, 1993).

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The LS is crucial for performance in the CPP paradigm (Sartor and Aston-Jones, 2012) and our anatomical and behavioural data strongly suggest that dLS GLP-1Rs contribute to this phenomenon. Although GLP-1R-deficiency is implicated in diminished hippocampus-dependent learning (During *et al*, 2003), and the CPP paradigm relies on contextual memory (Cunningham *et al*, 2006), we detected increased cocaine-induced conditioning of *Glp-1r*$^{-/-}$ animals providing evidence that the contextual impairment does not confound the interpretation of the CPP performance. Given that human addiction involves drug self-administration, future experiments could employ contingent models to examine the role of septal GLP-1Rs.

The neurochemical phenotype of GLP-1R expressing neurons is broadly unknown for most brain regions. We provide evidence that GLP-1R positive cells in the dLS are predominantly GABAergic and it is known that LS projections terminate in the VTA and NAc (Onteniente *et al*, 1987; Risold *et al*, 1997b; Swanson, 1982). Attenuated dopamine release in the NAc was reported following systemic Ex4 administration (Egecioglu *et al*, 2013a; Egecioglu *et al*, 2013c). Moreover, intra-VTA GLP-1R activation is mediated in part by non-glutamatergic mechanisms (Mietlicki-Baase *et al*, 2013). These data raise the possibility that the effects mediated by direct activation of GLP-1R in the mesolimbic system can be attributed to modulations on a circuit level. We propose that GLP-1R neurons in the dLS are essential for mesolimbic function. The underlying mechanisms are unclear but may involve downstream effects of GLP-1R signal transduction such as the modulation of cAMP levels (Drucker *et al*, 1987), activation of Ca^{2+}-channels (Acuna-Goycolea and van den Pol, 2004), or regulation of transcription factors (Klugmann *et al*, 2006).

In summary, we show that the dLS has the most prominent GLP-1 receptor expression in the brain. GLP-1R null mice exhibited increased excitability in GABAergic dorsal lateral septum neurons. These mutants had strongly augmented

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behavioural responses to cocaine when tested in two widely used non-contingent models of drug abuse. In a rescue experiment, we genetically complemented GLP-1R expression exclusively in the dLS of GLP-1R-deficient mice and observed restoration of cocaine-induced behaviour to wildtype levels. This dLS-directed rescue of the increased cocaine-induced behavioural measures in GLP-1R null mice indicates that GLP-1 transmission in this brain region is a key suppressor of addictive drug-induced behaviour. These findings will shift attention to this brain region and GLP-1 transmission as a novel target for therapeutic intervention in drug addiction.

Our working model (Figure 5) integrates our findings into a recently proposed CA3–dLS–VTA pathway that connects dLS activity to dopamine release from VTA neurons and, in turn, modulates motivated behaviour (Luo *et al*, 2011). Most GABAergic dLS neurons co-express *Glp-1r* mRNA (Figure 1). LS cells get activated by a range of stimuli (Sheehan, 2004), leading to context-dependent (Luo *et al*, 2011) and drug-induced adaptions of motivated behaviour. GLP-1R appears to play an essential role for these adaptations, suggested by our electrophysiological (Figure 2) and genetic rescue data (Figure 4B,C).

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Supplementary information is available at the *Neuropsychopharmacology* website.

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Figure legends:

Figure 1. *Glp-1r* mRNA is enriched in the lateral septum. (**A**) Illustration of a coronal mouse brain section. (**A_1-A_2**) *Glp-1r* mRNA expressing cells (purple) in the medial amygdaloid nucleus (red box in **A**) or the hypothalamus (blue box in **A**) labeled by arrowheads. (**A_3-A_4**) High magnification of individual *Glp-1r* mRNA positive cells. (**B**) Overview of *Glp-1r* mRNA expression (black staining) domains in the sagittal plane (Allen Brain Atlas ID 73606497_125). (**B_1-B_2**) Close ups of the NAc and the VTA showing little *Glp-1r* expression. (**C**) In the coronal plane highest *Glp-1r* expression (silver impregnation) was identified in the LS (Allen Brain Atlas ID 74511737_345). (D) The *Glp-1r* sense probe produced no signal. (**E**) Abundant *Glp-1r* mRNA expression (pink) was confirmed in the dLS using a specific DIG-labeled riboprobe. (**E_1**) Higher magnification of the dLS indicated by the box in (**E**) depicting strong cellular signal detection of *Glp-1r* mRNA. (**F**) Fluorescent detection of *Glp-1r* (red) and (**G**) *Gad65* (green) mRNA in the dLS. (**H**) Nuclear counterstaining with DAPI (blue). (**I**) Merged picture reveals that almost all *Glp-1r* positive septal neurons are GABAergic. MeA, medial amygdaloid nucleus; DMH, dorsomedial hypothalamic nucleus; Hippo, hippocampus; opt, optic tract; dLS, dorsal lateral septum; NAc, nucleus accumbens; LV, lateral ventricle; PG, pontine grey; VTA, ventral tegmental area. Bars: A_1, A_2, D, E, 75µm; A_3, A_4, E, F, 10µm; B, 1mm; B_1, B_2, 100µm; C, 200µm.

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Figure 2. Increased firing of *Glp-1r*$^{-/-}$ septal neurons. **(A)** The time to fire an AP is delayed in *Glp-1r*$^{-/-}$ (total of 23 cells from 10 animals) compared to *Glp-1r*$^{+/+}$ (total of 21 cells from 11 animals) neurons (P = 0.02). **(B)** The number of APs at twice threshold was elevated in *Glp-1r*$^{-/-}$ compared to *Glp-1r*$^{+/+}$ controls (P = 0.04). **(C)** Representative responses of *Glp-1r*$^{+/+}$ (left) and *Glp-1r*$^{-/-}$ (right) neuron, respectively,

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to 600 ms depolarizing current steps at 100 pA and 150 pA. **(D)** Responses (number of APs evoked by 600 ms stimulus) of *Glp-1r*$^{+/+}$ and *Glp-1r*$^{-/-}$ cells across a range of step current injections from 10 pA to 150 pA revealed an increased activity of *Glp-1r*$^{-/-}$ neurons (two-way repeated measures ANOVA x genotype effect: F(1,43) = 6.48, P = 0.01). *P < 0.05, **P < 0.01.

Figure 3. AAV-mediated *Glp-1r* gene delivery to the dLS. **(A)** AAV expression cassettes. ITR, inverted terminal repeat; CBA, chicken ß-actin promoter; pA, polyadenylation signal; WPRE, woodchuck hepatitis virus post-transcriptional regulatory element. (**B**) Biological activity of GLP-1R-GFP was confirmed by expression in heterologous HEK293 cells and exposure to Ex4 (10nM) for the indicated durations (0–50min). The dynamics of ERK phosphorylation were similar following activation of the tagged GLP-1R or the native receptor. Total ERK served as loading control. Note: The GFP-antibody exclusively recognized the tagged recombinant receptor. (**C**) Recombinant GLP-1R-GFP traffics to the cell surface in HEK293 cells and in the mouse LS (top row), while GFP controls show cytosolic expression (bottom row). Anti-GFP immunofluorescence (green); DAPI (blue) nuclear counterstain. Bars: 10µm. (**D**) Schematic illustration of vector spread assessed by AAV-transduced neuronal somata; cc, corpus callosum; dLS, dorsal lateral septum; LV, lateral ventricle.

Figure 4. Genetic complementation of GLP-1R expression in the dLS rescues cocaine-induced, but not anxiety-related, behaviours in *Glp-1r*-deficient mice. (**A**) *Glp-1r*$^{-/-}$ mice and *Glp-1r*$^{-/-}$ mice injected with AAV-GLP-1R-GFP spent less time in the centre of the EPM (left panel, one-way ANOVA F = 8.049; P = 0.0012); *Glp-1r*$^{+/+}$ controls (n = 17) compared with *Glp-1r*$^{-/-}$ mice (n = 15; P < 0.05), or *Glp-1r*$^{+/+}$ controls

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compared with *Glp-1r*$^{-/-}$ + AAV-GLP-1R-GFP (n = 9; *P* < 0.01). Middle panel; *Glp-1r*$^{-/-}$ mice and *Glp-1r*$^{-/-}$ mice injected with AAV-GLP-1R-GFP spent less time in the open arms (one-way ANOVA F = 14.34; *P* < 0.0001) compared with *Glp-1r*$^{+/+}$ controls (*P* < 0.0001), and entered the open arms less frequently (right panel; one-way ANOVA F = 9.65; *P* < 0.001; Post hoc test *P* < 0.01). (**B**) Altered locomotor response to cocaine for all groups over the course of the paradigm (two-way repeated measures ANOVA genotype effect F(2,41) = 9.771, *P* = 0.0003). There was no difference between genetically complemented *Glp-1r*$^{-/-}$ mice (n=9) and *Glp-1r*$^{+/+}$ controls (n = 17) following acute cocaine exposure (Day1; *P* > 0.9999). Intact development and expression of cocaine sensitization (two-way repeated measures ANOVA cocaine effect F(2,82) = 21.00, *P* < 0.0001) was present in *Glp-1r*$^{+/+}$ and *Glp-1r*$^{-/-}$ mice (n = 15) but absent in *Glp-1r*$^{-/-}$ + AAV-GLP-1R-GFP mice (Day1 - Day5, *P* = 0.5141 and Day1 - Day12, *P* = 0.2919). Note: There was no difference between *Glp-1r*$^{+/+}$ controls and *Glp-1r*$^{-/-}$ + AAV-GLP-1R-GFP on any given day. # represents *P* compared with Day 1; *represents *P* compared with all groups on a given day. (**C**) Genetically complemented *Glp-1r*$^{-/-}$ mice displayed a normal CPP phenotype (one-way ANOVA F = 7.037, *P* = 0.0023. The CPP score represents the difference between the time spent in the cocaine compartment before and following conditioning. * or # *P* < 0.05, ** or ## *P* < 0.01, ****P* < 0.001.

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Figure 5. Septal GLP-1R regulates motivated behaviour. GLP-1R expressing GABAergic septal neurons modulate cellular activity following stimulation. GLP-1Rs are present in both somata and terminals of these neurons (Göke, 1995; Shirazi *et al*, 2013) that form inhibitory synapses with GABAergic interneurons in the VTA (Luo *et al*, 2011). Their inhibition, in turn, leads to activation of dopaminergic VTA neurons via disinhibition. Hence, this dLS-VTA pathway controls drug-induced behaviours

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such as locomotor sensitization and drug-seeking. DA, dopamine. Adapted from Luo et al. (Luo *et al*, 2011).

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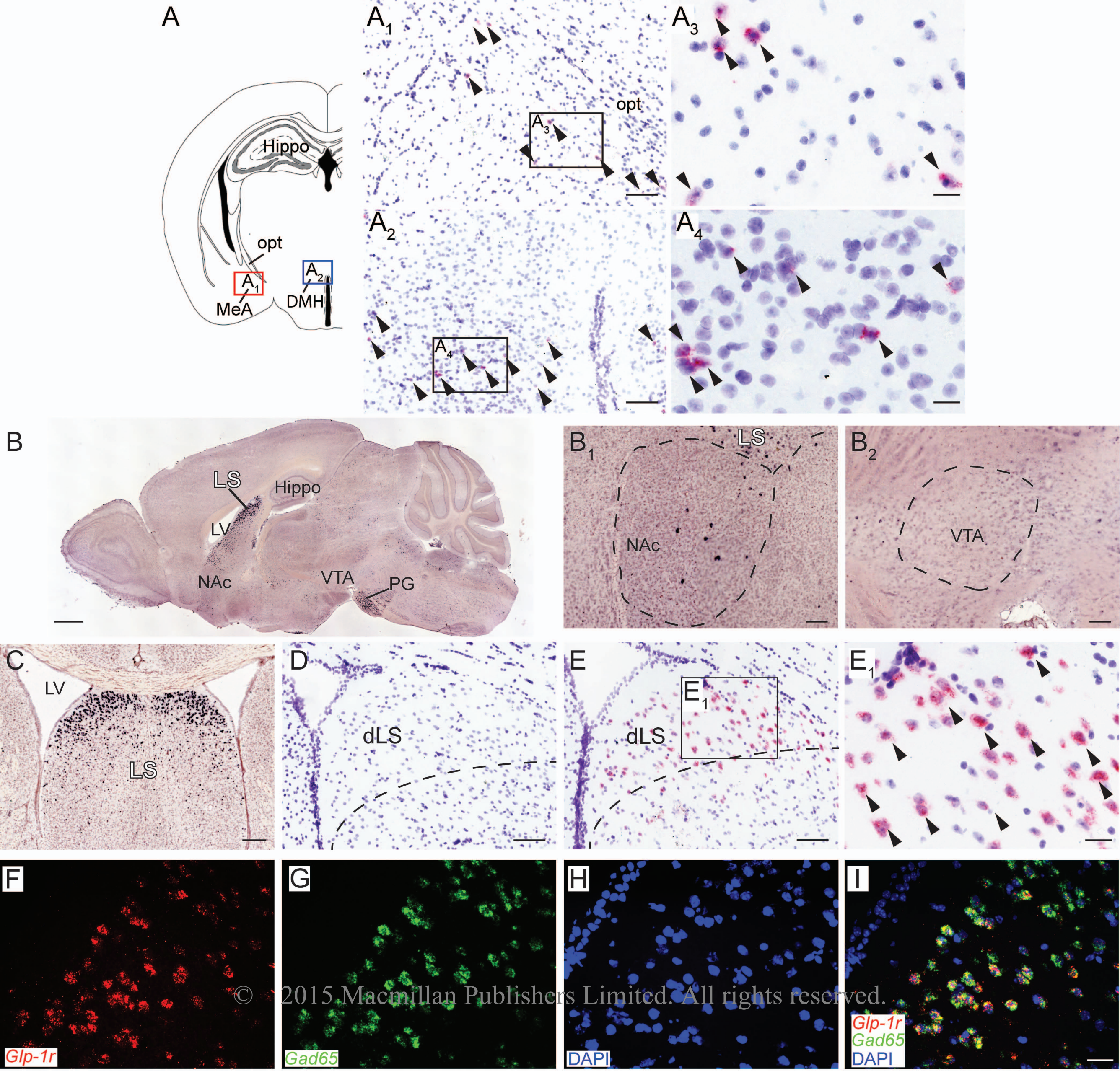

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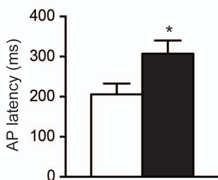

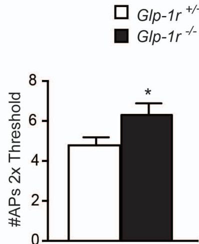

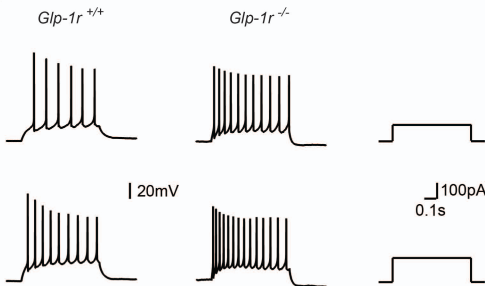

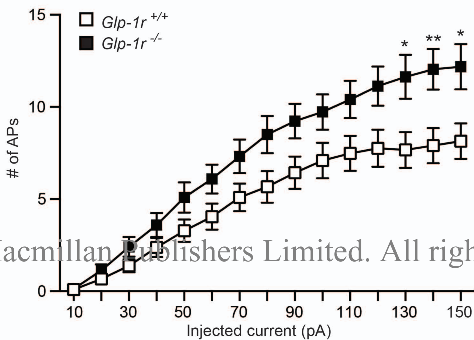

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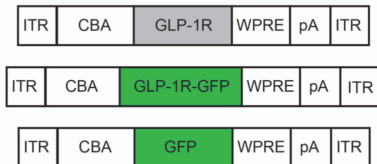

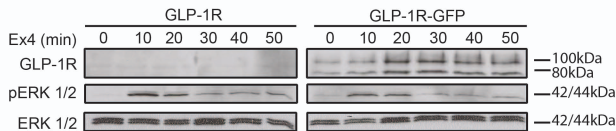

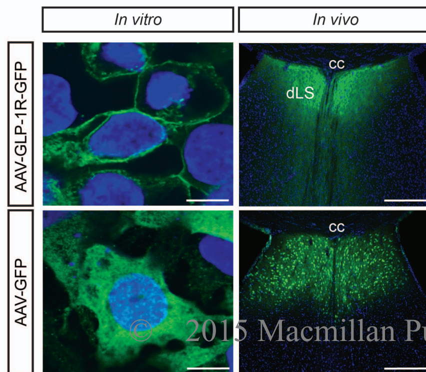

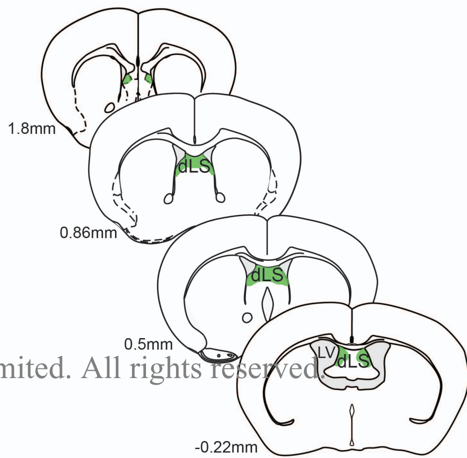

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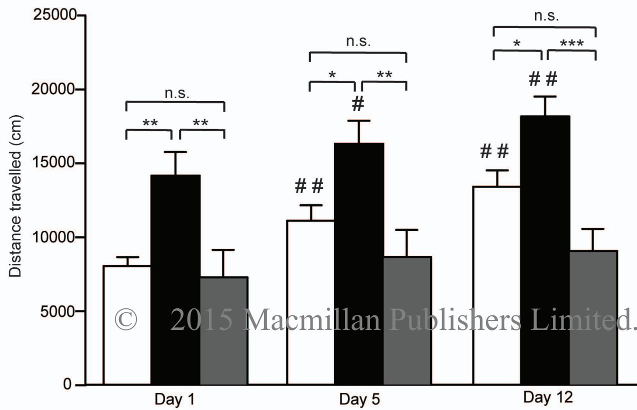
Glp-1r +/+
Glp-1r -/-
Glp-1r -/- + AAV-GLP-1R-GFP

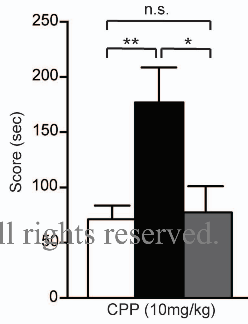
A

Time in Center (s)
Time in open arms (%)
Entries in open arm (%)

B



C



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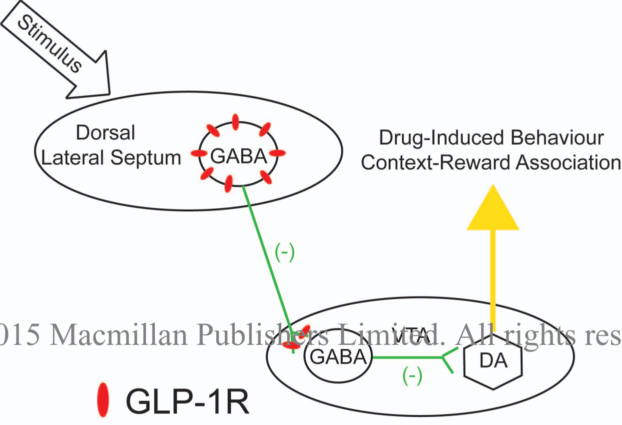

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