

## Viral-mediated delivery of an RXFP3 agonist into brain promotes arousal in mice

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### Summary

Anatomical and functional studies of central relaxin-3/RXFP3 systems suggest they constitute an ascending arousal network. For example, relaxin-3 knockout mice display circadian hypo-activity compared to wild type littermate controls. In studies to explore the effect of chronic RXFP3 activation on behaviour, we engineered a lentiviral construct to constitutively secrete the RXFP3 agonist, R3/I5, and express a green fluorescent protein (GFP) marker in transduced cells. Intracerebroventricular injection of the lenti-R3/I5-GFP virus (~10<sup>8</sup> genomic copies in 2  $\mu$ l) in adult C57BL/6J mice resulted in GFP expression within cells of the ventricle walls and choroid plexus over a period of 1-4 weeks, suggesting likely chronic R3/I5 secretion and RXFP3 activation in brain regions proximal to the ventricular system. Subsequent testing in automated locomotor cells on day 8 and 9 post-injection revealed that lenti-R3/I5-GFP treated mice displayed prolonged, elevated locomotor activity (~18% higher over the last 15 min on day 8, and over the entire 30 min test on day 9) compared to mice injected with a control lenti-GFP virus, which habituated normally to the novel environment (n=18/12 respectively, p<0.05). These findings are consistent with an earlier report of increased activity scores in rats acutely injected centrally with R3/I5, and further suggest a role for relaxin-3/RXFP3 signalling in promoting behavioural arousal.

### Key words

Relaxin-3, RXFP3, R3/I5, lentivirus, arousal, locomotor activity

### Introduction

Relaxin-3 is an abundant brain neuropeptide that binds and activates the  $G_{i/o}$ -protein coupled receptor, RXFP3 (Liu et al., 2003; Ma et al., 2007), and existing anatomical and functional evidence suggests relaxin-3/RXFP3 signalling modulates behavioural state and arousal (Smith et al., 2011). Arousal is broadly associated with a range of states including wakefulness, attention and vigilance; and influences modalities such as motivation, spatial navigation, and learning and memory. Relaxin-3/RXFP3 networks likely act via interactions with other circuits that play a role in promoting arousal. For example, high densities of relaxin-3 fibres and RXFP3 binding sites are present throughout the septohippocampal system (Ma et al., 2007; Smith et

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al., 2010), which strongly modulates hippocampal oscillatory activity, with increased theta rhythm considered the 'on' state of the hippocampus and functionally associated with exploratory behaviour (Vertes et al., 2004). Furthermore, relaxin-3 fibres/terminals and RXFP3 are abundant within the limbic system, which provides a major input to the hypothalamus and the mesolimbic dopaminergic pathway which are both important for motivation and reward drive; and there is accumulating functional evidence suggesting that relaxin-3/RXFP3 signalling can act on these processes, particularly feeding behaviour (Ganella et al., 2012, 2013).

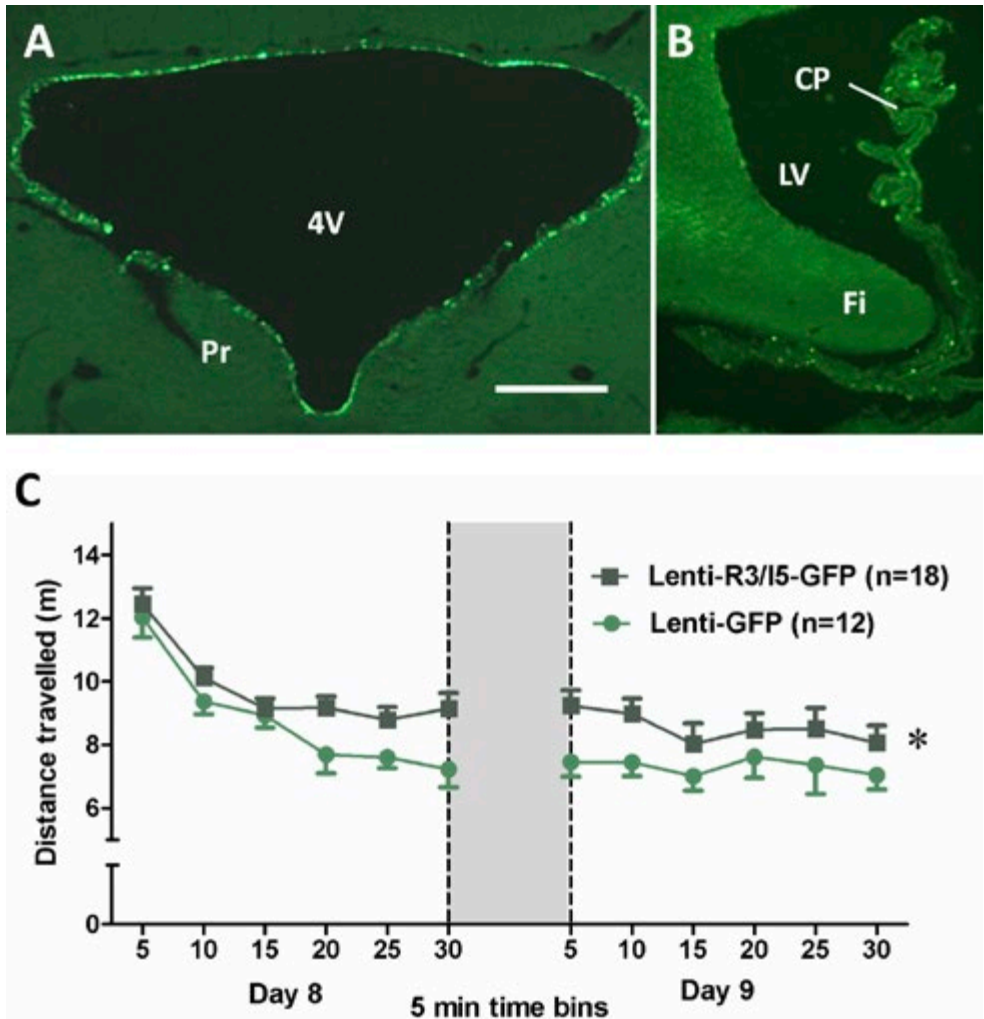
The aim of the present study was to further explore the role of relaxin-3/RXFP3 signalling in behavioural arousal by assessing the effect of chronic activation of RXFP3 in mice by the selective RXFP3 agonist *R3/I5* (Liu et al., 2005). Traditional methods of chronic peptide delivery, such as repeated injections or surgical implantation of a minipump and an indwelling brain cannula, have some disadvantages. For example, large quantities of peptides are required, and an indwelling minipump and cannula can be cumbersome and impede behaviour, while repeated handling for injections can obscure chronic behavioural changes. We recently developed an adeno-associated viral construct (AAV-FIB-R3/I5) that constitutively secretes the relaxin-3 agonist R3/I5 from transduced neurons, and which we demonstrated was effective in chronically modulating RXFP3 function in rat hypothalamus (Ganella et al., 2013). In this study we have cloned the FIB-R3/I5 coding region into lentivirus ('lenti-R3/I5-GFP') to enable the transduction of multiple cell types after central injection. Importantly, lentivirus injection into the lateral cerebral ventricle has been shown to transduce cells in the ventricle wall and choroid plexus, resulting in secretion of gene product into the cerebrospinal fluid and subsequent predictable biological actions (Regev et al., 2009).

## Methods

*Viral constructs:* The lenti-R3/I5-GFP construct consisted of (in 5' to 3' order): cytomegalovirus (CMV) promoter; FIB-R3/I5 coding region (FIB, fibronectin secretory sequence to ensure R3/I5 is secreted rather than retained in the cytoplasm; R3/I5, sequence encoding pro-R3/I5); internal ribosome entry site (IRES), and; sequence encoding GFP. The lenti-GFP construct consisted of a CMV promoter and the GFP sequence. *Mice and surgery:* Adult male C57BL/6J mice were anaesthetised and stereotactically injected with approximately  $10^8$  virulent particles in 2  $\mu$ l of either lenti-R3/I5-GFP or lenti-GFP (control) virus. *Automated locomotor cell:* On two consecutive days at 8 and 9 days post-surgery, mice were placed in automated locomotor cells (approx 40  $\times$  40 cm) under low lighting for 30 min during the light phase. *Histology:* Brains were harvested following transcardial perfusion, and 40  $\mu$ m sections were cut on a cryostat and mounted before imaging via fluorescence microscopy.

## Results

Following intracerebroventricular (icv) injection of lenti-R3/I5-GFP or lenti-GFP virus, GFP-positive cells were observed within the ventricle wall and choroid plex-



**Figure 1.** 1-4 weeks after icv infusion of the lenti-R3/I5-GFP virus in mice, transduced GFP-positive cells were observed within the ventricle wall (A) and choroid plexus (B) (two week time point is shown), indicating R3/I5 secretion into the cerebrospinal fluid over this time period. (C) Distance travelled in automated locomotor cells (30 min) by mice tested 8 and 9 days after infusion of either lenti-R3/I5-GFP or lenti-GFP virus. Abbreviations: 4V, 4th ventricle; CP, choroid plexus; Fi, fimbria of hippocampus; LV, lateral ventricle; Pr, prepositus nucleus. Scale bar = 300  $\mu$ m. Data are expressed as mean  $\pm$  SEM. \*  $p < 0.05$ , two-way RM ANOVA, main effect of treatment.

us 1-4 weeks post-injection (Fig. 1A,B; two week time point shown). Testing in automated locomotor cells on day 8 and 9 post-injection revealed that lenti-R3/I5-GFP virus-treated mice displayed prolonged, high levels of activity (i.e. reduced habituation) over the last 15 min on day 8, and over the entire 30 min on day 9, that was on

average 18% greater than the distance travelled by mice treated with the lenti-GFP control virus (two-way RM ANOVA, main effect of treatment,  $p < 0.05$ ; treatment  $\times$  time interaction,  $p > 0.05$ ) (Fig. 1C).

## Discussion and Conclusion

These initial studies demonstrate the likely utility of the lenti-R3/I5-GFP virus to chronically deliver R3/I5 into the brain of adult mice. This follows the successful use of the FIB-R3/I5 construct driven by AAV transduction in neurons of the rat brain to chronically modulate feeding (Ganella et al., 2013). Viral driven R3/I5 production therefore yields effective long term modulation of behaviour, although we are not yet able to directly measure R3/I5 levels in cerebrospinal fluid or brain, and hence it is difficult to determine dose-related effects. Nonetheless, the presence of a difference in the behaviour of mice injected with lenti-R3/I5-GFP virus compared to lenti-GFP controls suggests sufficient R3/I5 is present within the brain to activate RXFP3 within functionally relevant circuits.

The ability of chronic R3/I5 to reduce habituation to a novel environment and maintain higher locomotor activity during the test period is in line with previous studies of relaxin-3/RXFP3 function. For example, relaxin-3 knockout mice display circadian hypoactivity on running wheels compared to wildtype controls (Smith et al., 2012), and acute central infusion of R3/I5 in rats was reported to increase total activity in locomotor cells (Sutton et al., 2009) – an effect similar to that observed here. Furthermore, local infusion of a specific RXFP3 agonist into the rat medial septum was shown to promote hippocampal theta rhythm (Ma et al., 2009).

Intra-hypothalamic injection of AAV-FIB-R3/I5 was recently shown by our laboratory to increase bodyweight in rats (Ganella et al., 2013). A similar effect was absent in the present study (data not shown), which utilized a different experimental species (see Smith et al., this volume), injection site and virus/construct. Despite these differences, however, AAV-FIB-R3/I5-treated rats displayed a trend for increased activity compared to controls during their second session in automated locomotor cells, an effect in line with the reduced habituation observed in lenti-R3/I5-GFP-treated mice, which may reflect the modulation of homologous arousal-related circuits by R3/I5 in both studies.

In conclusion, the present study describes further evidence that relaxin-3/RXFP3 signalling acts to promote behavioural arousal in mice and provides an initial evaluation of the effectiveness of a new viral-based method to explore this emerging neuro-peptide/GPCR system.

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