Ligand-dependent temporal patterns of signalling and receptor phosphorylation at NMU2

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***Introduction*:** Neuromedins U (NmU) and S (NmS) mediate effects via Family A, $G\alpha_{q/11}$-coupled, G-protein-coupled receptors, NMU1 and NMU2. NMU2 is predominantly in the CNS suggesting it mediates central anorexigenic effects of NmU and/or NmS. We reported previously that NMU2 challenge with NmS for 5 min, followed by ligand removal, caused slower recovery of Ca^{2+} signalling and more sustained ERK signalling than activation with NmU, despite comparable initial Ca^{2+} responses (1). Here we examined p38 activation and, to consider potential differences in NMU2 phosphorylation and links to signalling differences, we employed *C*-terminally HA-tagged NMU2 (NMU2-HA) to determine temporal profiles of ligand-dependent receptor phosphorylation and de-phosphorylation.

***Method*:** HEK-293 cell lines with stable expression of NMU2 or NMU2-HA were used to determine ERK activation, p38 activation and receptor phosphorylation using established methods (2).

Results: Initial experiments demonstrated NMU2-HA behaved similarly to NMU2. Thus, immunoblotting phosphorylated ERK (pERK) to indicate activation showed equivalent potency of the ligands at the two receptors (pEC_{50} 5 min: 8.56±0.08 and 8.49±0.11 for NmU and NmS respectively at NMU2; 8.70±0.08 and 8.90±0.08 for NmU and NmS at NMU2-HA; mean±sem, n≥3). Consistent with our previous data using NMU2 (1), challenge of NMU2 or NMU2-HA with NmU (30 nM, 5 min) followed by ligand removal evoked rapid activation of ERK that subsided over 3 h. In contrast, NmS (30 nM, 5 min) ERK activation was sustained until at least 3 h. Immunoblotting of phospho-p38 indicated equivalent potency of NmU and NmS on NMU2-HA-mediated p38 activation (pEC_{50} 5 min: NmU, 8.77±0.23; NmS, 8.91±0.19). Following NmU (30 nM, 5 min) and ligand removal, p38 activity subsided to basal by 3 h, whereas following NmS (30 nM) activation was more sustained. Immunoprecipitating NMU2-HA from ^{32}P-labelled-cells and subsequent autoradiography demonstrated significant increases in receptor phosphorylation by the two ligands (1 µM, 5 min, ~7-11-fold over basal) with NmS significantly greater. Challenge followed by ligand removal caused more prolonged receptor phosphorylation following NmS (63±8% maximum at 90 min) than NmU (basal at 90 min).

Conclusion: These data show ligand-dependent differences in MAPK signalling by NMU2 using a protocol that may be similar to transient exposure *in vivo*. Further, this protocol revealed ligand-dependent temporal patterns of NMU2 phosphorylation, suggesting this may contribute to ligand-dependent differences in receptor signalling and processing.

References:

1. Bahattab O *et al.* (2014). *Proceedings of the British Pharmacological Society* http://www.pa2online.org/abstracts/Vol12Issue1abst043P.pdf
2. Prihandoko R *et al*. (2015). *Curr Protoc Pharmacol* **69**: 11-26.