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## INTRODUCTION

Melanin-concentrating hormone (MCH) is a nineteen amino acid cyclic neuropeptide derived from pre-pro MCH (Nahon, 1994). To date, two human MCH receptors have now been identified, MCH<sub>1</sub> (SLC-1) and MCH<sub>2</sub> (Chambers et al., 1999; Saito et al., 1999; Hill et al., 2001). There is a considerable body of evidence indicating a role for MCH in the control of food intake and energy balance: icv administration of MCH to rats increases food intake (Qu et al., 1996; Rossi et al., 1999); MCH mRNA is overexpressed in *ob/ob* mice and fasted mice (Qu et al., 1996); MCH overexpressing mice are hyperphagic, mildly obese, hyperglycaemic and insulin resistant (Ludwig et al., 2001); MCH knockout mice are leaner than wild-type mice due to hypophagia and increased metabolic rate (Shimada et al., 1998). The MCH<sub>1</sub> receptor appears to mediate the orexigenic effects of MCH (Chambers et al., 1999). Numerous MCH<sub>1</sub> receptor antagonists for the potential treatment of obesity have appeared in recent years with at least two compounds entering clinical development (Mendez-Andino and Wos, 2007).

Previously iodinated ligands have been used to label MCH<sub>1</sub> receptors. Here we describe the development and validation of an in vitro radioligand receptor binding assay for MCH<sub>1</sub> receptors using  $[^3\text{H}]\text{AMR-MCH-1}$  and human cloned MCH<sub>1</sub> receptors. Using a modification of this assay the ex vivo occupancy of striatal MCH<sub>1</sub> receptors by SCH-A has been determined in dietary-induced obese C57BL/6J mice using the technique of autoradiography.

## METHODS

### RECEPTOR BINDING METHODOLOGY

**Membrane preparation:** MCH<sub>1</sub> membranes (Batch 1138 Euroscreen, protein concentration 4.4 µg/ml) were resuspended in ice-cold 50 mM Tris-HCl, pH 7.4 and placed on ice for 5 minutes prior to use in the binding assay.

**Saturation binding studies:** Membranes (400µl; equivalent to 8.92µg tissue/tube) were incubated with 50µl of  $[^3\text{H}]\text{AMR-MCH-1}$  (8 concentrations 0.25 – 16 nM) and 50µl of incubation buffer (total binding) or 50 µl of SCH-A (50 µM; non-specific binding) at 25°C for 60 minutes.

**Recovery of membrane-bound radioactivity:** Membrane-bound radioactivity was recovered by rapid filtration under vacuum through Skatron 11731 filters pre-soaked in 0.5% polyethylenimine, using a Skatron cell harvester (setting 9,9,0). Filters were then washed with ice-cold 50mM Tris-HCl, pH 7.4.

### PRELIMINARY EXPERIMENTS

**Effect of tissue concentration:** Binding of  $[^3\text{H}]\text{AMR-MCH-1}$  (~1/10 Kd, Kd and 10 x Kd) to MCH<sub>1</sub> receptors was evaluated using a range of tissue concentrations (2.23, 4.46, 8.92 and 13.37 µg/tube).

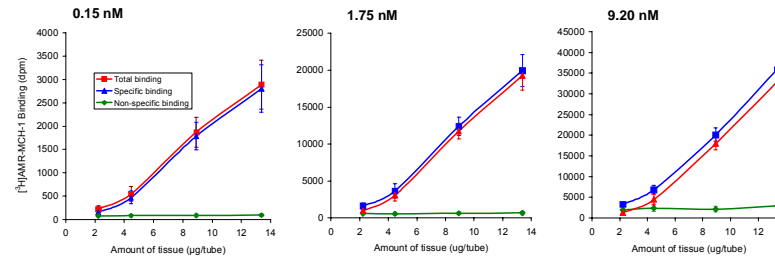
**Effect of incubation time:** The binding of  $[^3\text{H}]\text{AMR-MCH-1}$  (~1/10 Kd, Kd and 10 x Kd) to MCH<sub>1</sub> receptors was evaluated over different time periods (1, 2, 5, 10, 15, 20, 30, 45, 60, 75, 90, and 120 minutes).

**Data analysis:** Equilibrium dissociation constants (Kd) and the maximal number of binding sites (Bmax) were determined by non-linear regression analysis.

### EX VIVO

Mice were given vehicle (po) or SCH-A (10 or 30 mg/kg po) and terminated 6 or 24 hours later. Brains were removed. Coronal sections containing the caudate putamen were cut. Slides were incubated with  $[^3\text{H}]\text{AMR-MCH-1}$  (1.4 nM) and 50 mM Tris-HCl buffer, pH 7.4 (total binding) or SCH-A (50µM; non-specific binding) at room temperature for 60 minutes. Binding was terminated by aspiration and sections washed in ice-cold buffer (4 x 15 mins). Radioactivity bound to the section was determined using a Beta- imager.

Fig 1. Effect of tissue concentration on  $[^3\text{H}]\text{AMR-MCH-1}$  binding to MCH<sub>1</sub> receptors



Tab 1. Effect of tissue concentration on  $[^3\text{H}]\text{AMR-MCH-1}$  (1.75nM) binding to MCH<sub>1</sub> receptors

Tissue up per tube	$[^3\text{H}]\text{AMR-MCH-1}$ binding at 1.75 ± 0.13 nM				
	NSB (dpm)	TB (dpm)	SB (dpm)	SB/TB%	TB/RA%
2.23	340	939	600	63.8%	0.8%
	1044	2781	1738	62.5%	1.6%
	735	1568	833	53.1%	1.0%
Mean ± SEM	627 ± 164	1618 ± 409	991 ± 254	62% ± 3	1% ± 2
4.46	229	1308	1079	82.5%	1.1%
	1245	5324	4579	78.0%	3.4%
	531	4480	3949	88.1%	2.7%
Mean ± SEM	589 ± 227	3660 ± 970	3071 ± 773	84% ± 2	2% ± 0.5
8.92	346	9756	9410	96.5%	8.2%
	1116	15383	14267	92.7%	9.1%
	746	13495	12750	94.5%	8.3%
Mean ± SEM	659 ± 175	12414 ± 1257	11755 ± 1086	95% ± 1	8% ± 0.5
13.37	250	14923	14673	98.3%	12.5%
	1202	25639	24437	98.3%	15.1%
	894	19753	18859	95.5%	12.1%
Mean ± SEM	691 ± 218	19971 ± 1295	19280 ± 2000	97% ± 1	13% ± 1

## RESULTS

Binding of  $[^3\text{H}]\text{AMR-MCH-1}$  (0.15, 1.75 and 9.2 nM) increased linearly with protein over the range 4.46 to 13.37 µg protein/tube (Figure 1). A protein concentration of 8.92 µg protein/tube was chosen. Under these conditions specific binding was good (96±1% 0.15nM, 95±1% 1.75nM (Table 1) and 89±3% 9.2nM, respectively; mean ± SEM, n=4) and the total radioactivity bound as a percentage of the radioactivity added was (14±1% 0.15nM, 8±0.5% 1.75 nM (Table 1) and 2.5±0.2% 9.2nM, respectively; mean ± SEM, n=4). Binding (0.1, 1 and 9.5 nM) reached equilibrium within 30 minutes and remained constant for up to 2 hours (data not shown). Therefore an incubation time of 60 minutes was chosen. Full saturation binding analysis revealed that binding approached saturation at the highest concentrations of radioligand. Binding was of high affinity and fitted well to a single site binding model (Kd = 1.42±0.08 nM and Bmax = 13.3±0.7 pmoles/mg protein; mean ± SEM, n=4; Figure 2).

Specific binding of  $[^3\text{H}]\text{AMR-MCH-1}$  was high in the caudate putamen of brains from dietary-induced C57BL/6J mice in the vehicle-treated group, with low levels of non-specific binding as defined by SCH-A (Figure 3A). SCH-A (10 and 30 mg/kg po) occupied 95% and 102% at 6 hours and 46% and 78% at 24 hours post-dose, respectively of striatal MCH<sub>1</sub> receptors when compared to controls (all p<0.001; n=5 mice per group; Figure 3B).

## SUMMARY

**These data indicate that  $[^3\text{H}]\text{AMR-MCH-1}$  is a high affinity, selective ligand (Sargent et al., this meeting) which can be used to label recombinant and native MCH<sub>1</sub> receptors. The ex vivo binding data is in good agreement with Kowalski et al., 2006.**

## REFERENCES

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Fig 2. Typical Saturation and Scatchard binding plots for  $[^3\text{H}]\text{AMR-MCH-1}$  binding to MCH<sub>1</sub> receptors

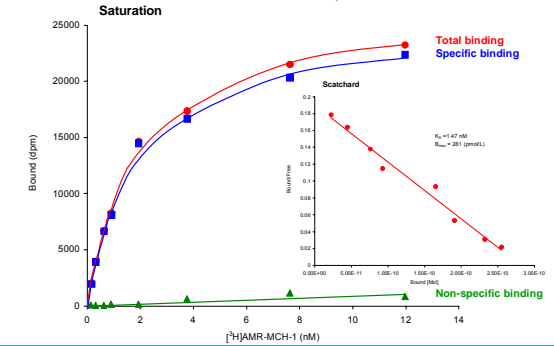


Fig 3A.  $[^3\text{H}]\text{AMR-MCH-1}$  ex vivo autoradiography in caudate putamen of diet-induced obese mice 6 H and 24 H following oral administration of SCH-A (10 and 30 mg/kg).

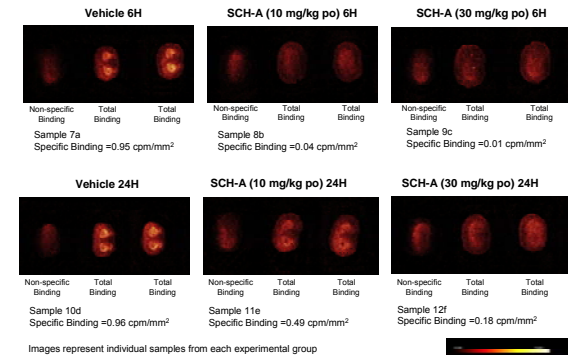
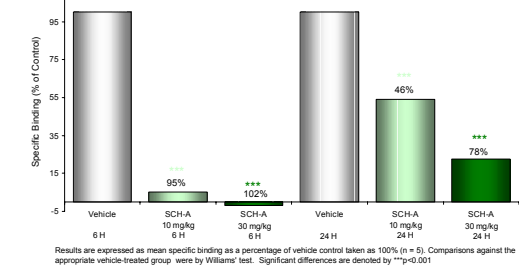


Fig 3B.  $[^3\text{H}]\text{AMR-MCH-1}$  ex vivo autoradiography in caudate putamen of diet-induced obese mice 6 H and 24 H following oral administration of SCH-A (10 and 30 mg/kg).



Results are expressed as mean specific binding as a percentage of vehicle control taken as 100% (n = 5). Comparisons against the appropriate vehicle-treated group were by Wilcoxon test. Significant differences are denoted by \*p<0.001