Is Narcolepsy an Antibody-mediated Autoimmune Disease?





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A	Symptoms of Excessive Daytime Sleepiness (EDS) for more than 3 months
B	Definite history of cataplexy: episodes of sudden, transient loss of muscle tone precipitated by emotion
C	Confirmation by CSF Orexin-A measurement which should be less than or equal to 110 pg/ml OR Confirmation by Multiple Sleep Latency Testing following nocturnal polysomnography: Following a minimum of 6 hours sleep, mean sleep latency should be less than 8 minutes, and at least 2 Sleep Onset REM Periods (SOMREMPs) should be observed, defined as the electroencephalographic appearance of REM sleep within 40 minutes of sleep onset.
D	The hypersomnia is not better explained by another cause, including other sleep disorders, neurological or psychiatric disorders, medication or substance misuse

Table 1.1: ICSD-2 Diagnostic criteria for narcolepsy-cataplexy, 2005

Test	**Sensitivity**	**Specificity**
HLA DQB*0602	89.3% (822/1291)	76 (1291)
CSF orexin <110 pg/ml	83.3% (182/233)	>99%
MSLT	87.9% (964/1095)	96.9% (1095)

Table 1.2: **Sensitivity and specificity of tests for narcolepsy-cataplexy**
(Adapted from Mignot 2011)

Table 1.3: Evidence for and against autoimmunity in narcolepsy

	For	Against
Epidemiology	Environmental influences indicated by teenage/adult age of onset (Dauvilliers et al. 2001), March peak and September trough in birth pattern (Dauvilliers et al. 2003), twin discordance (Hublin et al., 1994) Association with streptococcal infections (Aran et al., 2009) and H1N1 vaccination (Dauvilliers et al. 2010)	Unlike many autoimmune diseases, the sex ratio in narcolepsy is similar (Silber, Krahn, Olson, & Pankratz, 2002) No clustering of other autoimmune diseases is reported in narcoleptics (Rubin, Hajdukovich, & Mitler, 1988)
Treatment Trials	Several case reports found favorable effects of immunosuppression in early stages of the disease (e.g. Dauvilliers et al. 2004 and 2009)	Other case reports found no benefit of intravenous immunoglobulins close to disease onset (e.g. Knudsen et al., 2012)
Biomarkers of disease	ASO titers correlated with decreased disease duration (Aran et al., 2009)	Narcolepsy onset is not associated with peripheral markers of inflammation such as elevated CRP (Aran et al., 2009) Oligoclonal bands are rare and no reported increase in IgG index (Schuld, Uhr, & Pollma, 2004)
Autoantibodies	Autoantibodies detected against Tribbles homologue-2 (Cvetkovic-lopes et al., 2010; Kawashima et al., 2010; Toyoda et al., 2010) Case reports of narcolepsy-cataplexy patients with antibodies against known neurological autoantigens, including NMDA receptor, Aquaporin-4 and Ma-2 (Adams, Mckeon, Silber, & Kumar, 2012; Baba et al., 2009; Tsutsui et al., 2012) Neurological autoantigens such as LGI1 colocalise with orexin neurons (Irani et al., 2012)	No evidence for autoantibodies against orexin or its receptors, the most obvious targets No increase in autoimmune-associated antibodies including ANA, DNA, SS-A, RF and SM (Rubin et al., 1988)
T cells	Tight association with HLA DRB2 and DQB1*0602 (Honda et al. 1983; Mignot 1998) GWAS studies identified polymorphisms in T cell receptor alpha (Hallmayer et al., 2009) and the T cell-expressed purinergic receptor P2Y11 (Kornum et al., 2011)	Although some gliosis is reported, autopsy studies have not demonstrated the typical hallmarks of T cell autoimmunity, including the accumulation of activated CD4+ cells and macrophages, up-regulation of MHC II and cytokines (Thannickal et al. 2003)

Table 1.4: **Candidate autoantigens in narcolepsy-cataplexy**
Usual clinical features of autoimmune condition and predicted immunofluorescence (IF) and western blot (WB) appearances

Candidate Autoantibody	Antigen	Clinical Features	IF	WB
NMDAR (Vincent et al 2011)	NR1 (mainly) and NR2b subunits of the NMDA glutamate receptor, thought to be associated with synaptic plasticity and memory	Causative autoantibody in some cases of cortico-subcortical encephalopathy. Associated with ovarian teratomas present in 50%	High signal in hippocampal neurophil	Polyclonal antibodies against linearised epitopes give bands at 116 and 180 kDa (NR1 and NR2b respectively) (Garcia-Gallo et al. 2001)
CASPR2 (Vincent et al, 2011)	VGKC-associated Caspr2	Causative autoantibody in some cases of encephalitis (Morvan's phenotype)	High signal in hippocampal and cerebellar neurophil	180 kDa protein (Poliac et al). Unknown WB appearance
LGI1 (Vincent et al, 2011)	VGKC-associated LGI1	Causative autoantibody in some cases of encephalitis (limbic phenotype)	Widespread expression High signal in hippocampal neurophil	Bands at 60 and 70 KDa (Santa Cruz, Sc28238)
CONTACTIN2 (Vincent et al, 2011)	VGKC-associated CONTACTIN2	Encephalitis	Unknown	Band at 150kda (Santa Cruz, sc-376780)
GABA A R a3	a3 subunit of the GABA A receptor. Ligand-gated chloride channels are formed with pentamers consist of alpha, beta and gamma subunits (Bell et al, 1989)	None known	Subunit specific pattern in hypothalamus including orexin neurons (Backberg et al, 2004)	50 kDa band (Sigma)
TRIB2	Ubiquitously expressed member of the protein kinase superfamily. Cytoplasmic localisation (UniProt Knowledgebase)	Associated with anterior uveitis (Zhang, 2005). High Trib-2 titres associated with narcolepsy-cataplexy close to disease onset (Cvetkovic-Lopes et al., 2004).	Widespread expression	Band at 37 kDa (AB73547)

N.B.: Vincent et al. 2011 is a review. All other refs are primary papers

Table 1.5: Other autoantigens tested in this study
Predicted immunofluorescence (IF) and western blot (WB) appearances

Autoantibody	Antigen	Clinical features	IF	WB
Hu (ANNA 1) (Hughes et al, 2008)	Family of similar proteins (splice variants). Role in neuronal development. Nuclear and cytoplasmic localisation.	Paraneoplastic encephalomyelitis SCLC in 80%.	CNS and PNS Neuronal nuclei, nucleolar sparing, weak cytoplasm	Wide band around 40kDa used to confirm IF pattern in diagnosis. (Santacruz Sc28299)
Yo (PCA-1) (Hughes et al, 2008)	Ribosomes, RER and Golgi apparatus of Purkinje cells. 3 Cerebellar degeneration related (CDR) proteins with CDR 2 as the major autoantigen: cerebellar, brainstem, intestinal mucosa	Subacute cerebellar ataxia in ovarian and breast carcinomas (90%)	Purkinje cell cytoplasm, nucleus sparing	3x CDR proteins: bands at 34, 52 and 62 kDa used to confirm IF pattern in diagnosis
AQP4 (Hughes et al, 2008)	The major water channel protein of the CNS. Transmembrane protein concentrated on astrocyte foot processes of the Blood Brain Barrier	Causative autoantibodies the demyelinating condition neuromyelitis optica	Staining of the pia, sub-pia and Virchow-Robin spaces lining blood vessels	Polyclonal antibodies raised against linearised epitopes give a prominent band around 35 kDa (Santacruz sc-9887)

N.B. Hughes et al., 2008 is a review

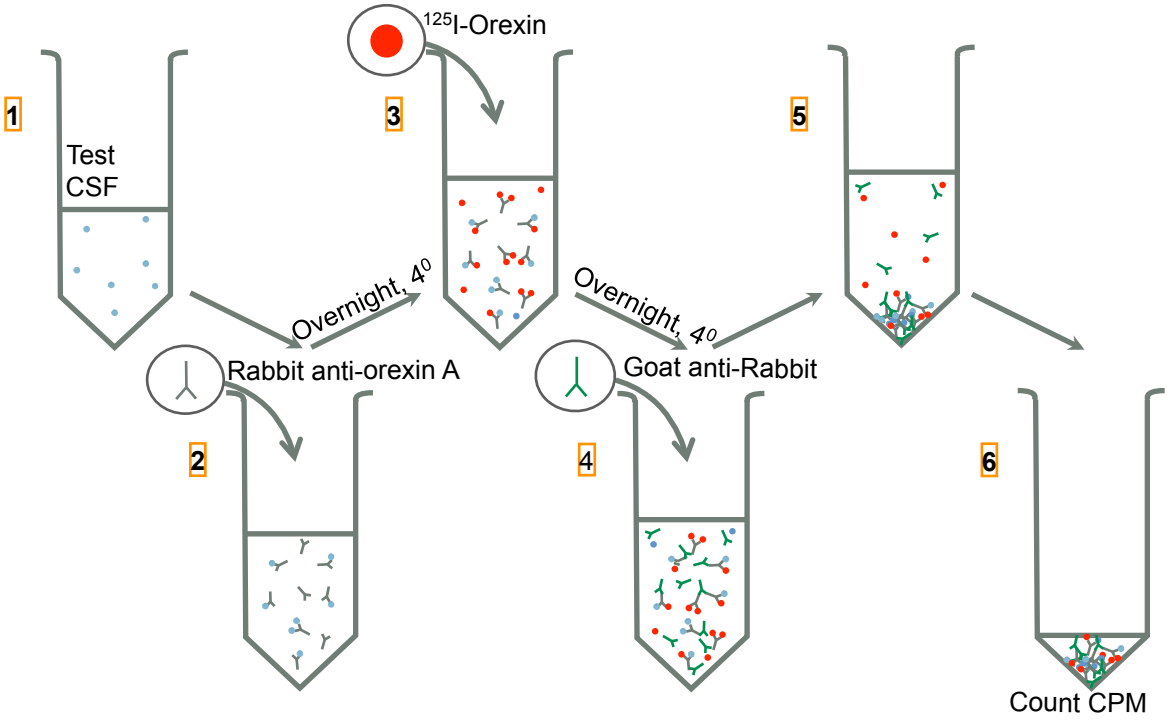


Figure 2.2: Summary of CSF orexin A RIA method
100 µl of test CSF, with an unknown quantity of orexin A peptide (represented by blue circles, (**1**), was diluted in RIA buffer and an orexin A antibody added rabbit anti orexin A: grey, (**2**). This solution was incubated overnight at 4ºC prior to the addition of a known concentration of ^{125}I-orexin A (red circles, (**3**). Orexin-anti orexin complexes were then immunoprecipitated by adding a secondary antibody (goat anti-rabbit: green, (**4**) prior to centrifugation (**5**) and supernatant aspiration (**6**). Radioactivity of the resultant pellet could then be counted.

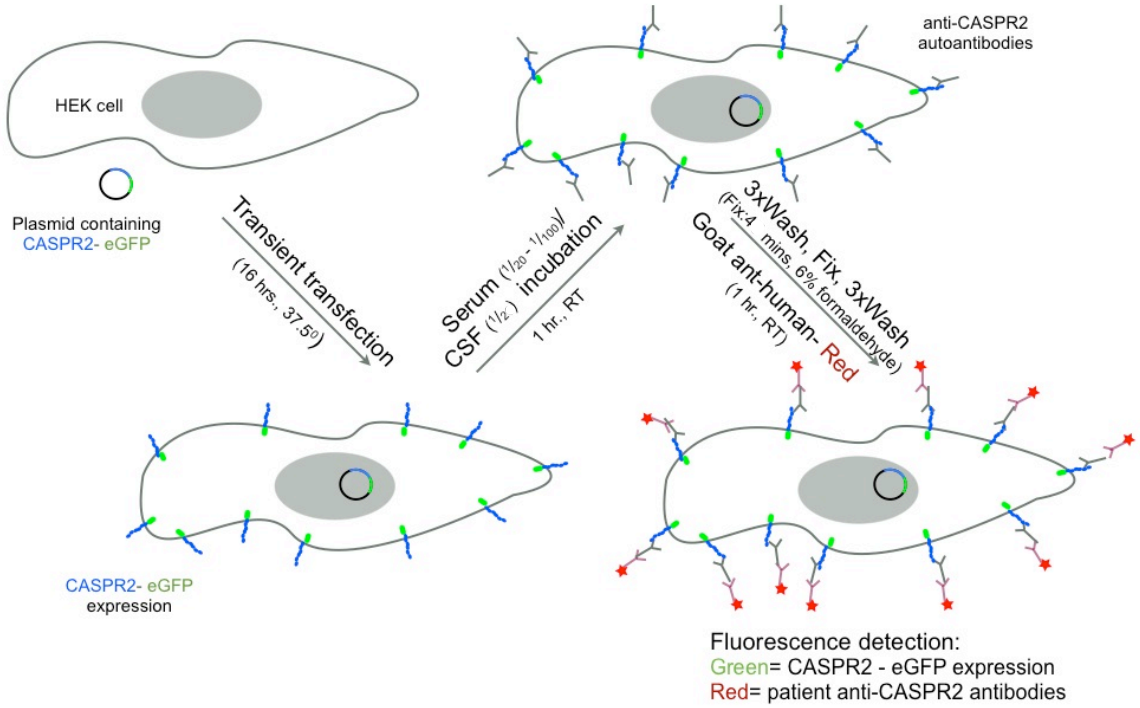


Figure 2.3: Summary of CBA method
HEK cells were transfected with with plasmids coding for respective antigens of interest. Following a period of growth to allow cell surface expression of the candidate antigen (in this case tagged with eGFP), live cells were incubated with patient serum/CSF prior to fixation. Patient antibody binding to the conformational antigen could then be assessed by the addition of a fluorescently-labelled secondary antibody and subsequent fluorescence microscopy

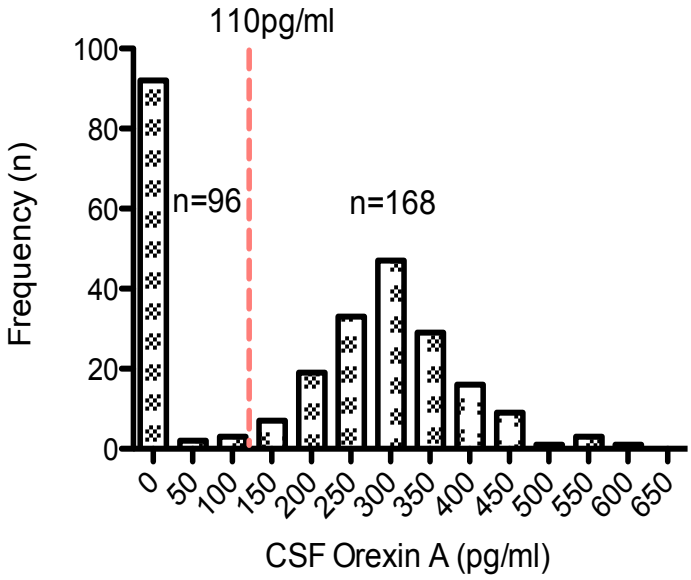
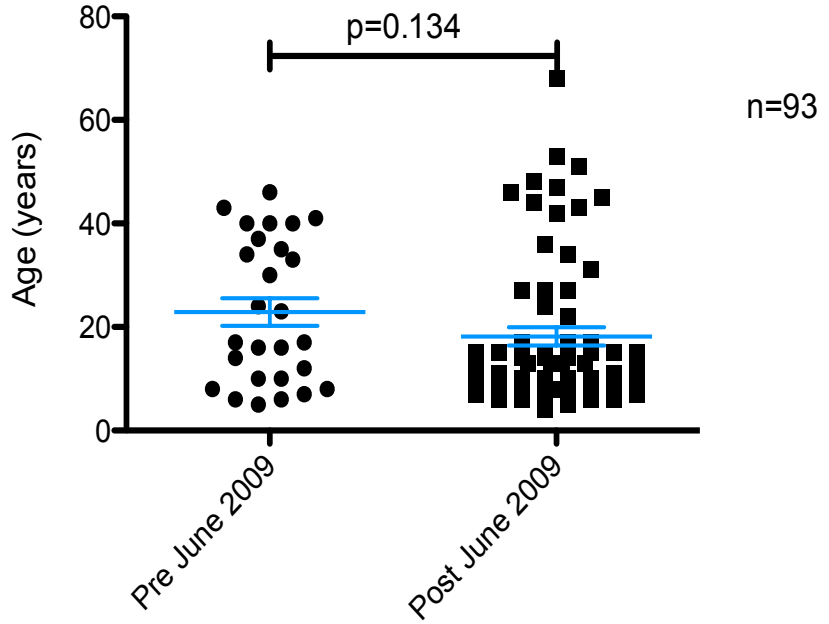


Figure 3.1: **Frequency distribution of CSF orexin measurements performed at the NDCN since 2006:** Using the diagnostic threshold of 110 pg/ml, 96 patients were orexin deficient and 168 patients had normal levels. Most orexin-deficient patients had undetectable levels (<40pg/ml). Orexin measurements in patients with normal levels fitted a Gaussian distribution. 132 of these samples were from patients aged less than 18 years old, 65 of which were orexin-deficient.



	Children	Adults
Post June 2009	50	16
Pre June 2009	14	13

*p=0.0291

Figure 3.2: **Ages of orexin deficient patients tested at the NDCN pre and post June 2009:** Patient age was available for 93/96 orexin-deficient CSF samples tested at the NDCN. Swine flu was reported in the UK in mid 2009. Ages of orexin deficient tested patients before and after June 2009 are plotted (mean ± SEM). Comparison by non-parametric unpaired t test did not reveal significant differences. However, comparison of groups by contingency table and fisher's exact test shows significantly greater proportion of orexin-deficient children tested since Swine Flu and subsequent vaccination.

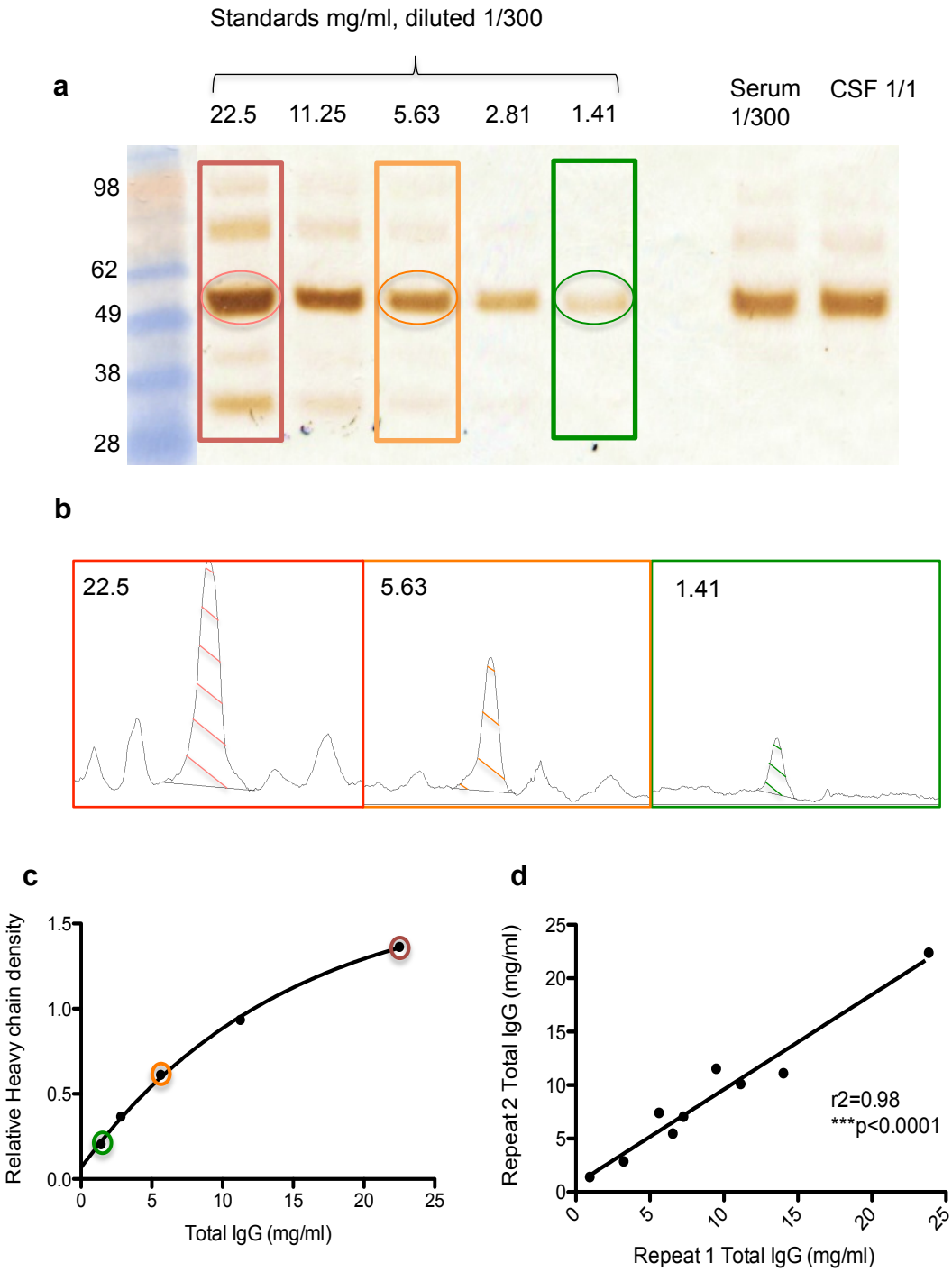


Figure 3.3: **Quantification of Total IgG in sera/CSF by densitometric analysis of DAB stained western blots**. 9.75ul of 1/300 standard/serum or neat CSF of were loaded per well. The IgG heavy chain was easily identifiable as the darkest band at around 50kDa (**a**). For each lane, The density of this band was quantified using the ImageJ Analyze->Blots plugin to calculate the area under the 50kDa peak above background (**b)**. Heavy chain densities were made relative to duplicate 11.25 mg/ml standards on each blot, and total IgG interpolated from a standard curve fitted with a One-phase exponential decay function (**c**). Total IgG values for CSF samples were divided by 300 to account for relative dilutions of standard/ serum (1/300) and CSF (neat). To minamise inaccuracies arising from colourmetric saturation at high IgG concentrations, any sample with a Total IgG measurement of more than 11.25 mg/ml was repeated at 1/3 dilution of the previous sample (1/900 serum, 1/3 CSF). Samples repeated on separate Western Blots with different standard curves showed significant correlation, confirming the reproducibility of this method (**d**).

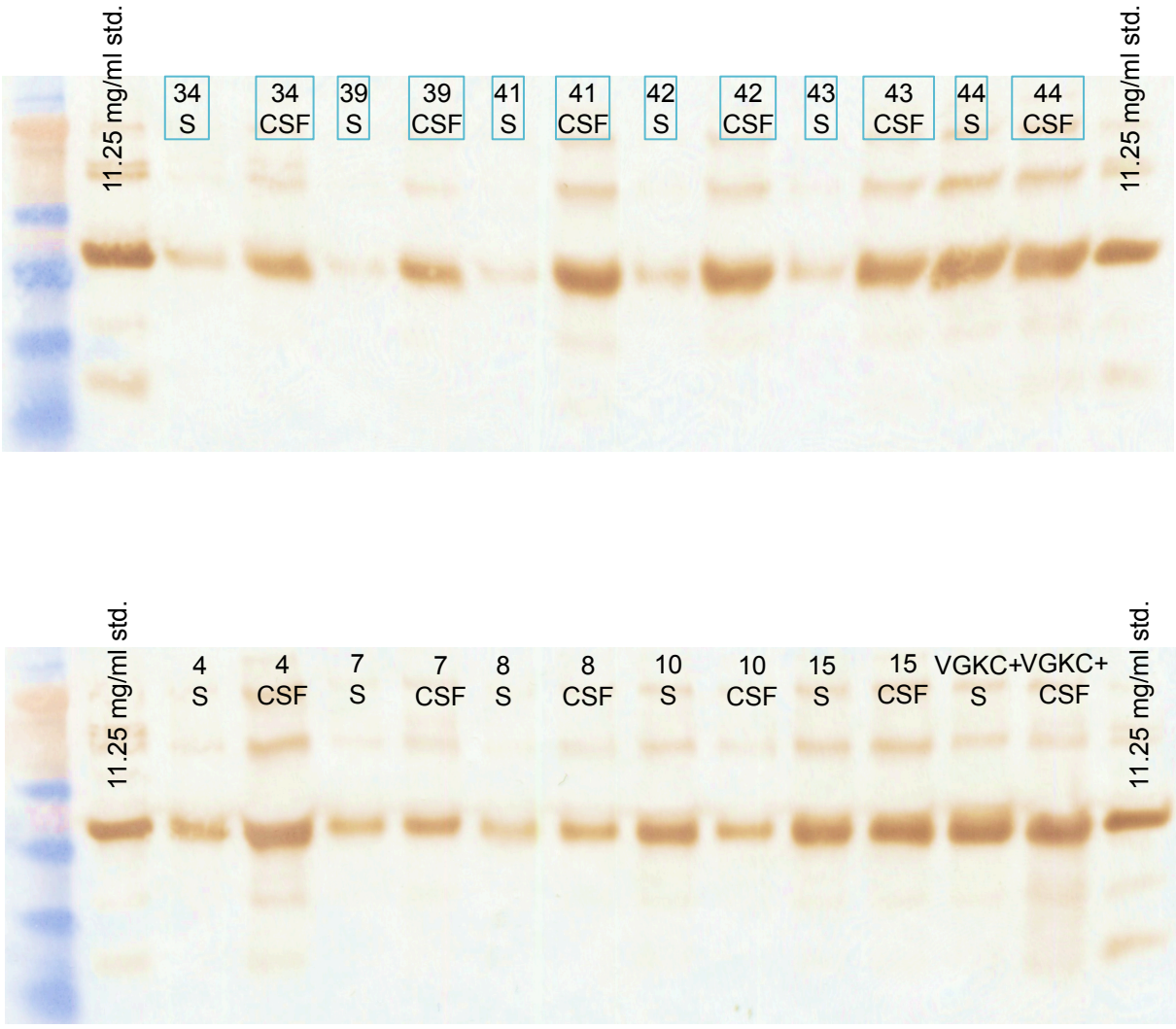
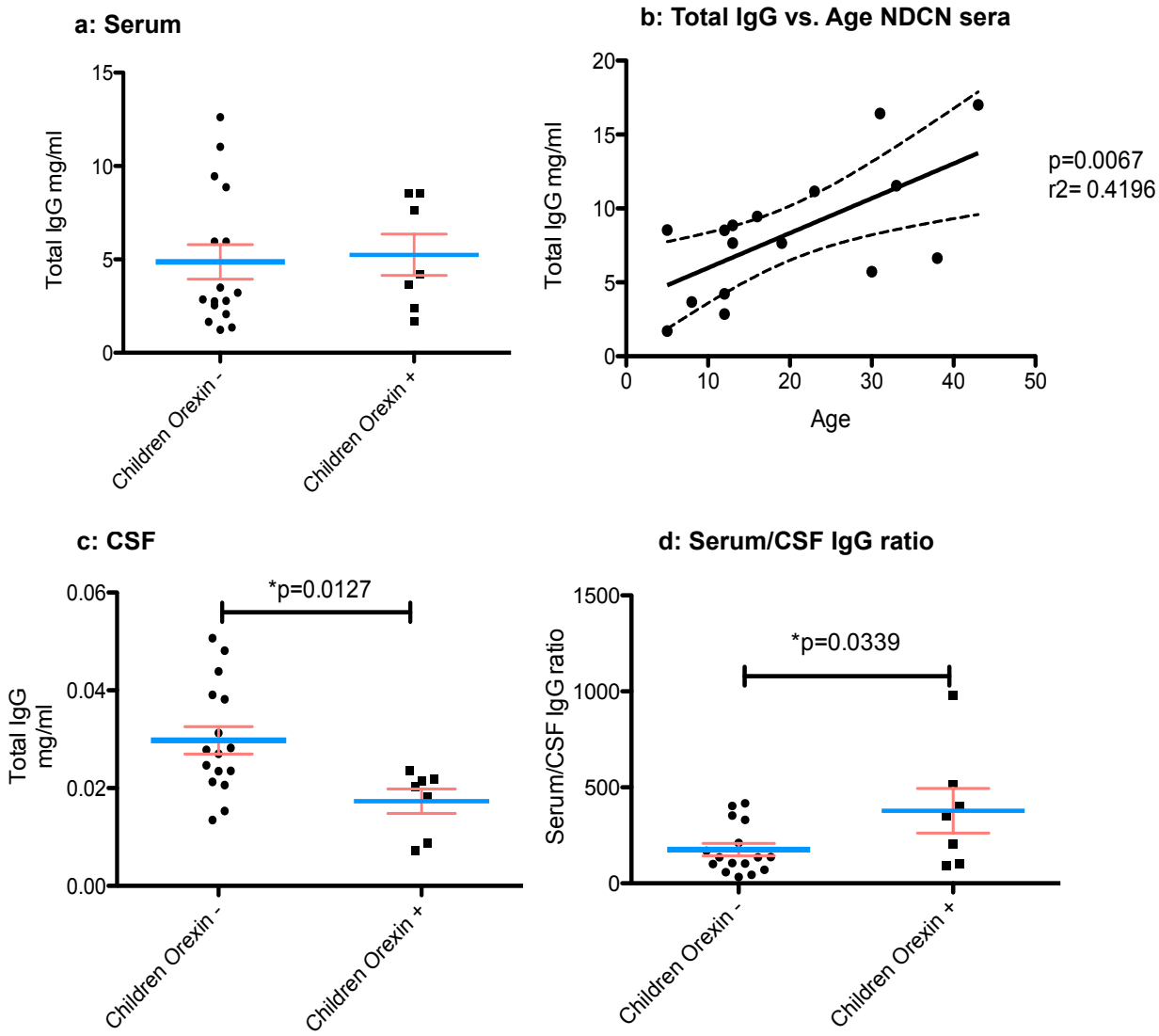


Figure 3.4: Representative western blots of serum/CSF Total IgG:
11.25 mg/ml IgG standards were included at ether end of each blot enabling relative quantification of the 50kDa IgG heavy chains. Serum (1:300 dilution, “S”) and CSF (undiluted) pairs are displayed (sample number also displayed). Samples from orexin-deficient children are highlighted in blue boxes. Some of the orexin deficient children appeared to have low serum IgG compared to respective CSF’s.



e: Total IgG reference ranges

Age	Serum IgG (mg/ml)	CSF IgG (mg/ml)	Serum/CSF IgG ratio
1-6	2.2-11.3	?	?
6-12	2.6-15.2		
12-18	5.2-15.6		
Adults	6.05-15.4	<0.06	>312.5

Figure 3.5: **Total IgG in sera and CSF of orexin-deficient vs. orexin-normal children**
a: Serum Total IgG in children. There was no difference between mean total serum IgG observed in orexin-deficient and orexin-normal children (unpaired T test)
b: Total IgG vs age NDCN sera. Age was the most important factor in determining Total IgG, regardless of orexin status
c: CSF Total IgG in children. CSF IgG was significantly higher in orexin-deficient compared to orexin-normal children; in several cases, more than a 2 fold elevation was observed.
d. Serum/CSF IgG ratio: Orexin-deficient children had a statistically significantly lower serum/CSF IgG ratio. This indicate that these children had elevated CSF levels relative to the serum
e. Age-stratified total IgG Reference Ranges in serum and CSF. Total IgG in children's sera was not significantly elevated except in one sample. Curiously, 6 samples had levels below the lower limit of this reference range. The lack of pediatric reference ranges made CSF IgG and serum/CSF IgG ratio difficult to interpret, although values for serum/CSF IgG ratio in these children was often lower than the quoted reference values found in adults. (Meulenbroek and Zeijlemaker, 1996; Thompson, 2005)
NB: Mean ± SEM are plotted for all comparisons. "Orexin –" = Orexin-deficient. "Orexin +" = Orexin-normal

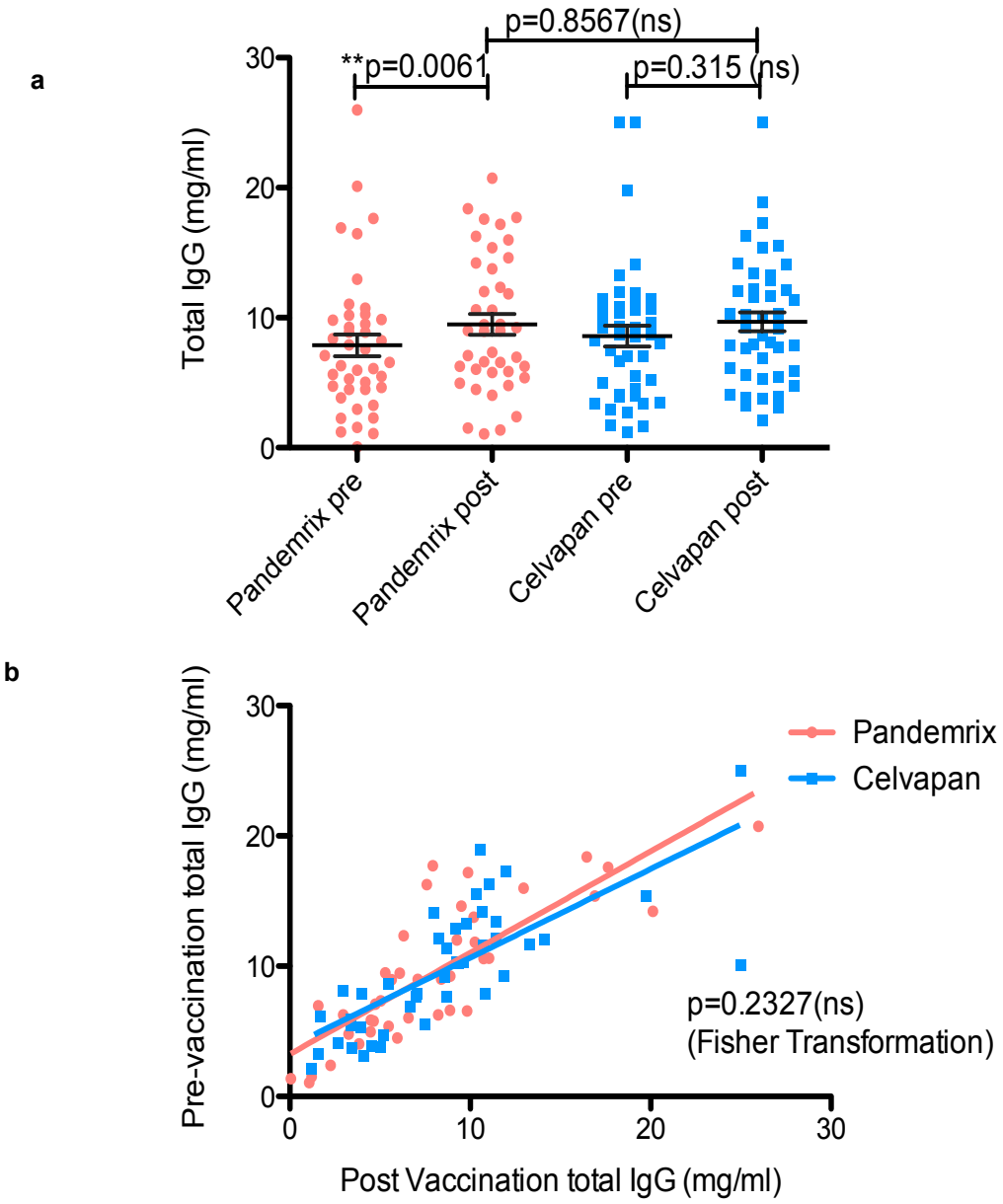


Figure 3.6: **Serum total IgG in a cohort of healthy control children pre and post H1N1 vaccination**

a: Total IgG pre and post vaccination. With Pandemrix®, total IgG was found to be slightly but significantly increased following vaccination. These differences were not significant for Celvapan®. Comparison of post vaccination total IgG levels between the vaccines did not reveal significant differences.

(Statistical comparison: pre vs. post vaccination total IgG comparisons=Paired T-test. Pandemrix® post vs. Celvapan® post vaccination total IgG= Unpaired T-test)

Figure 3.6: Serum total IgG in a cohort of healthy control children pre and post H1N1

a: Total IgG pre and post vaccination with Pandemrix® and Celvapan®:

using the Fisher Transformation there was no significant differences in total

Pandemrix

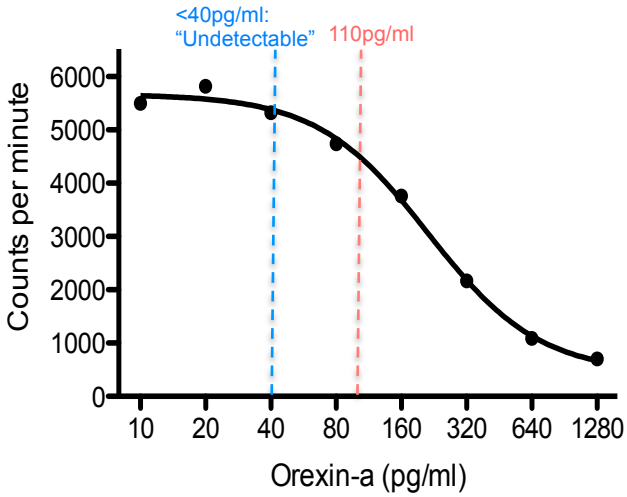
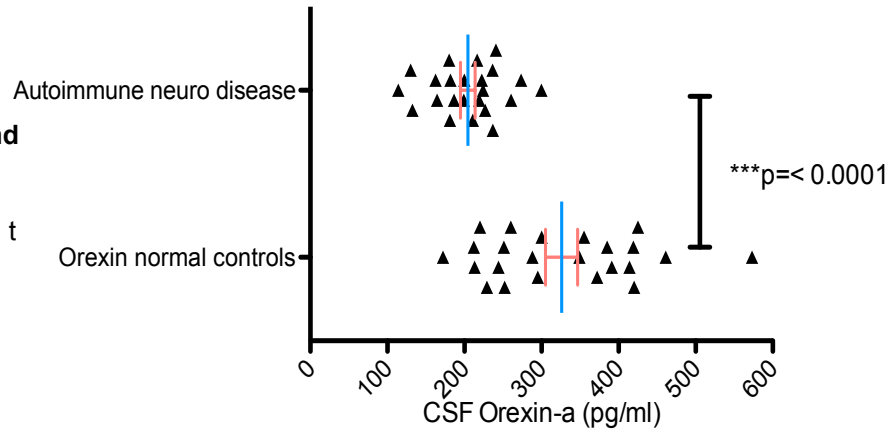


Figure 3.7: Representative example of a CSF orexin assay standard curve
By interpolating counts per minute onto the standard curve fitted with a sigmoid dose response non linear-regression model, sample orexin A could be quantified in pg/ml. Less than 110 pg/ml is considered the diagnostic threshold for narcolepsy cataplexy. Less than 40 pg/ml is considered below the assay's limit of detectability. Levels between 110 and 200pg/ml are considered intermediate, although in this study, all measurements >200pg/ml are considered normal.

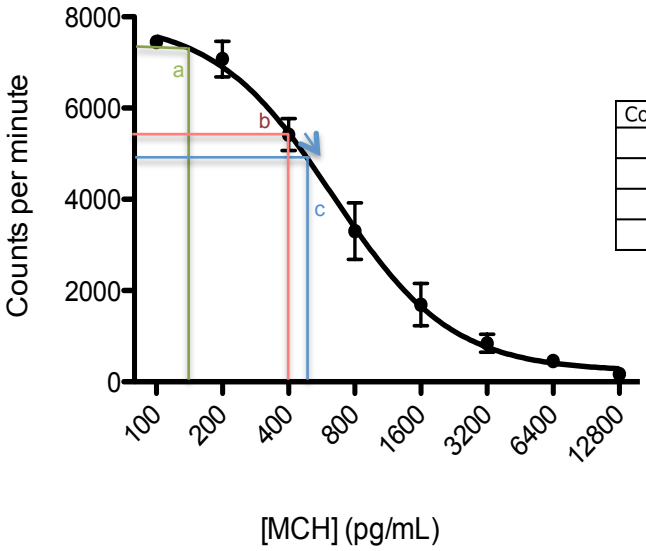
Figure 3.8: **CSF orexin levels in 23 autoimmune neurological disease patients compared with 23 randomly-selected and re-tested orexin normal patients** (mean ± SEM) Comparison with unpaired t test revealed significant differences between the group means



Antibody	Serum?	CSF?	Sex	CSF Orexin-a (pg/ml)
AQP4	y	y	M	130.3
AQP4	y	y	F	186.6
AQP4	y	y (low)	F	199.9
AQP4	y	y	F	210.7
AQP4	y	y	F	219.2
AQP4	y	y	F	224.2
AQP4	y (low)	y (low)	F	236.5
AQP4	y	y	F	240.8
GlyR	y	y (low)	M	199.3
GlyR	n	y	M	236.8
NMDAR	y	y	F	164.5
NMDAR	y	y	F	273.6
VGKC	y	n	F	132.5
VGKC	y	y (low)	M	162.6
VGKC	y	n	M	216.5
VGKC	y (low)	n	M	222.4
VGKC	y (low)	n	M	260.5
VGKC	y (low)	n	F	299.9
Undefined	-	-	M	114.3
Undefined	-	-	F	180.1
Undefined	-	-	F	181.1
Undefined	-	-	M	181.8
Undefined	-	-	F	226.9

Table 3.2: CSF Orexin levels in 23 autoimmune neurological disease patients grouped by autoimmune status
Levels did not correlate with a particular autoantibody. The lowest levels were found in a patient without a defined autoantibody.

Abbreviations: y=antibodies detected in serum/CSF; n=antibodies not detected in serum/CSF; APQ4: Aquaporin 4 antibodies in NMO patients; GlyR: Glycine receptor antibodies; NMDA receptor antibodies in encephalitic patients; VGKC= Voltage-gated potassium channel antibodies, usually found in patients with Limbic encephalitis and Morvan's syndrome. Undefined = patients tested negative for autoantigens, suspected autoimmune disorder.



Counts per minute	MCH (pg/ml)	
3951.20	263.37	Mean:
4174.40	217.31	247.55
3795.20	297.69	Standard dev:
4202.00	211.84	40.63

Figure 3.9: **Triplicate standard curve of MCH radioimmunoassay** (mean ± SD). It was not possible to accurately interpolate phisiological CSF MCH levels; the standard curve had saturated by this point (a). Therefore, samples intentionally spiked with 400pg/ml of MCH by adding 12.5µl of 3.2µg/ml standard peptide to each 100µl sample (b). Sample MCH could then be calculated by subtracting 400pg/ml from the observed MCH level (c).
Repeat measurement of one sample with sufficient CSF volumes is displayed alongside

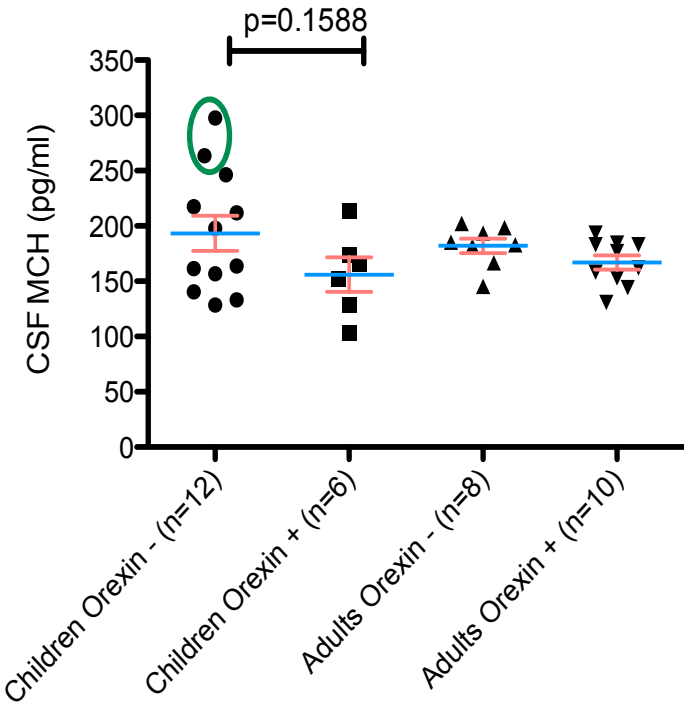
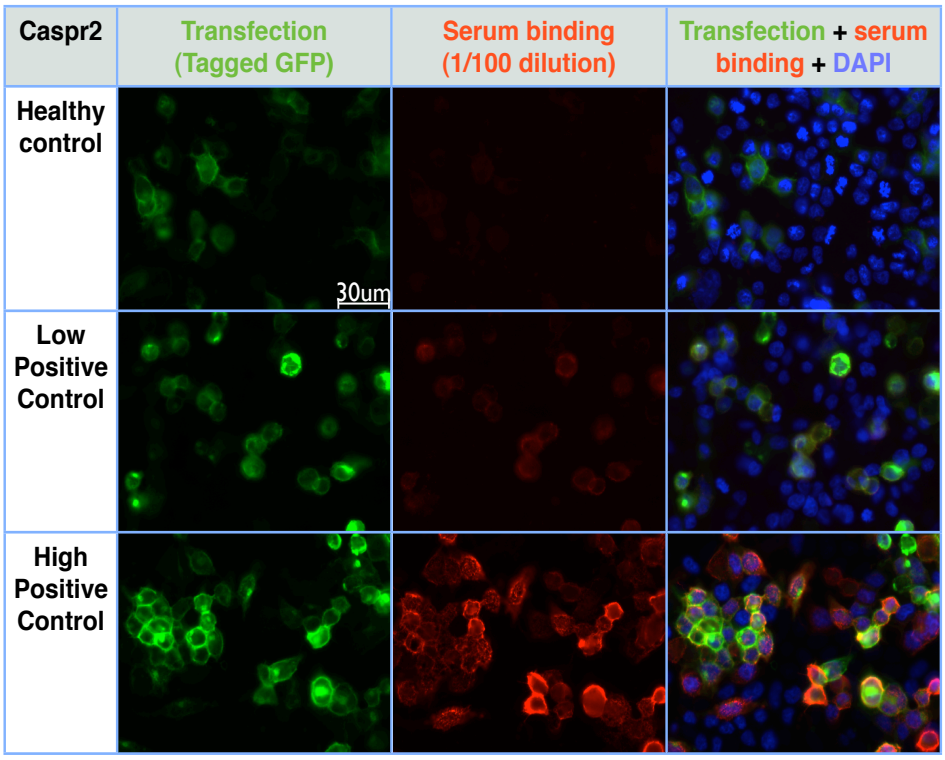


Figure 3.10: **CSF MCH measurements grouped by age and orexin status.** There was no significant difference in mean MCH measurements **(**mean ± SEM) . However, the 4 highest MCH levels were found in orexin deficient children. Two had levels more than 2 SD above the population mean (circled in green).
N.B.: "Orexin –" = Orexin deficient. "Orexin +" = Orexin normal

		Clinical Data		Biomarkers				
		Sex	Age	neuropeptides (pg/ml)		IgG (mg/ml)		
	No			Orexin a	MCH	Serum	CSF	IgG ratio
sera/CSF Orexin - Children	30	F	7	10	-	-	-	-
	18	F	8	10	-	-	-	-
	16	M	10	10	-	-	-	-
	33	M	10	10	-	-	-	-
	3	M	12	10	-	2.9	0.027	105.7
	53	F	13	10	-	8.9	0.021	416.3
	29	F	14	10	-	-	-	-
	57	M	16	10	-	9.5	0.023	402.7
sera/CSF Orexin + Children	13	F	5	363	-	1.7	0.018	94.3
	55	M	5	288	-	8.5	0.009	979.7
	56	M	8	217	-	3.7	0.007	514.3
	8	F	12	177	213	4.2	0.020	207.3
	54	M	12	265	-	8.5	0.021	399.0
	12	F	13	301	-	7.7	0.022	351.8
	5	F	15	262	-	-	-	-
sera/CSF Orexin + Adults	20	M	19	199		7.7	0.022	351.8
	28	F	19	385	131	-	-	-
	14	M	20	387	162	-	-	-
	24	F	20	516	153	11.2	0.013	858.7
	27	F	21	343	-	-	-	-
	25	F	23	250	144	-	-	-
	9	F	24	276	-	-	-	-
	22	M	28	287	-	-	-	-
	6	M	29	162	184	-	-	-
	2	F	31	223	183	16.4	0.072	228.1
	10	F	33	300		11.5	0.016	727.8
	52	M	33	300	-	-	-	-
	4	M	38	194	158	6.6	0.044	149.5
	15	M	43	333	194	17.0	0.083	204.1
	19		44	264	-	-	-	-
	17		44	268	-	-		-
	21	F	57	242	183	-	-	-
vaccination narcolepsy-cataplexy	36	M	4	10	-	6.0	0.028	211.0
	39	F	8	10	-	1.4	0.013	100.9
	40	F	8	-	-	-	-	
	37	M	9	52	-	11.0	0.031	352.9
	38	F	9	290	-	2.4	0.024	101.0
	41	M	11	10	141	1.7	0.051	32.6
	42	M	12	10	263	2.8	0.048	57.8
	44	M	14	10	-	12.6	0.038	330.4
	35	F	15	10	133	3.5	0.021	169.6
	34	F	16	10	-	3.2	0.024	137.0
	43	M	16	10	217	2.8	0.039	70.4
vaccination narcolepsy-cataplexy	48	F	9	-	-	-	-	-
	47	F	12	-	-	-	-	-
	49	F	13	-	-	-	-	-
	51	M	17	10	298	1.2	0.028	44.4
	45	M	18	10	246	6.0	0.044	135.7
	50	F	18	10	-	2.5	0.025	103.4
	46	M	24	10	198	2.1	0.015	135.3

NB "-" = Insufficient sample volume remaining to be tested

a: EGFP-tagged antigens: CASPR2, LGI1



b: Untagged antigens: NMDAR, CONTACTIN2

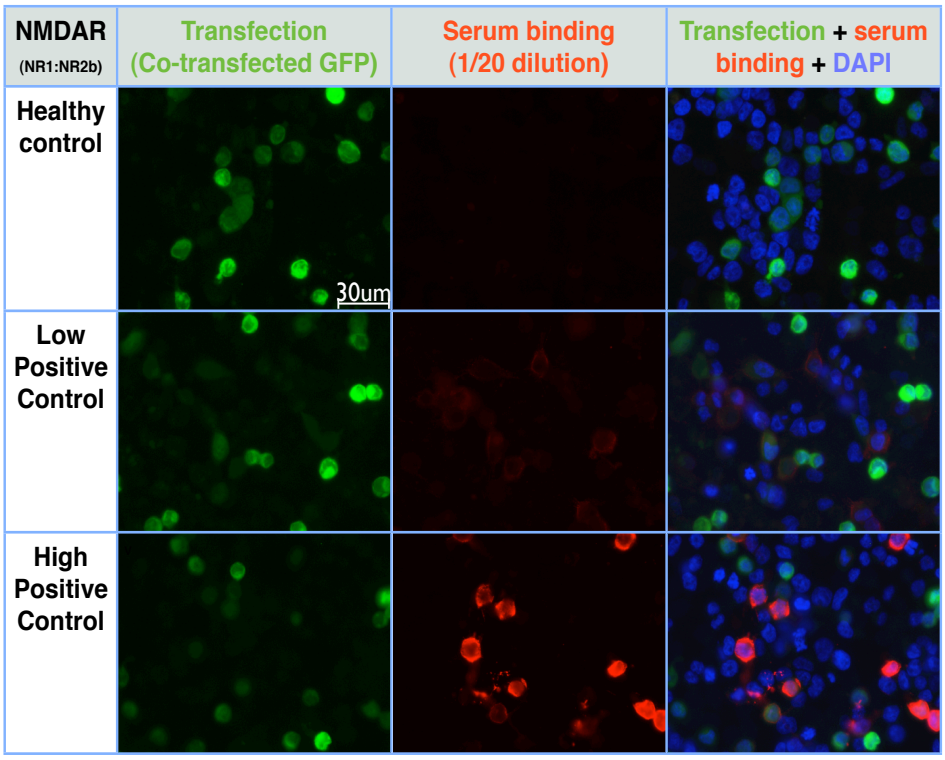


Figure 4.1: **Representative panel of CBA positive and negative controls.** Subjective CBA scoring in the CBA was decided by comparison with a panel of positive and negative controls. Scores (0-4) were given on the basis of the number of transfected cells with co-localisation and the intensity of the binding observed (o= no binding, 4=intense binding to all cells). Positive scores (>1) were confirmed by reassessment of coverslips by a blinded observer and re-testing in a separate assay.

	Children			Adults				
	Orexin -	Orexin +	Healthy	Orexin -	Orexin +	Healthy	CBA+ Controls	Total
Sera	24	5	5	6	17	10	5	n=72
CSF	29	9	0	17	15	0	0	n=70

Table 4.1: Randomised samples tested on NMDAR, CASPR2, LGI1 and CONTACTIN2 cell-based assays

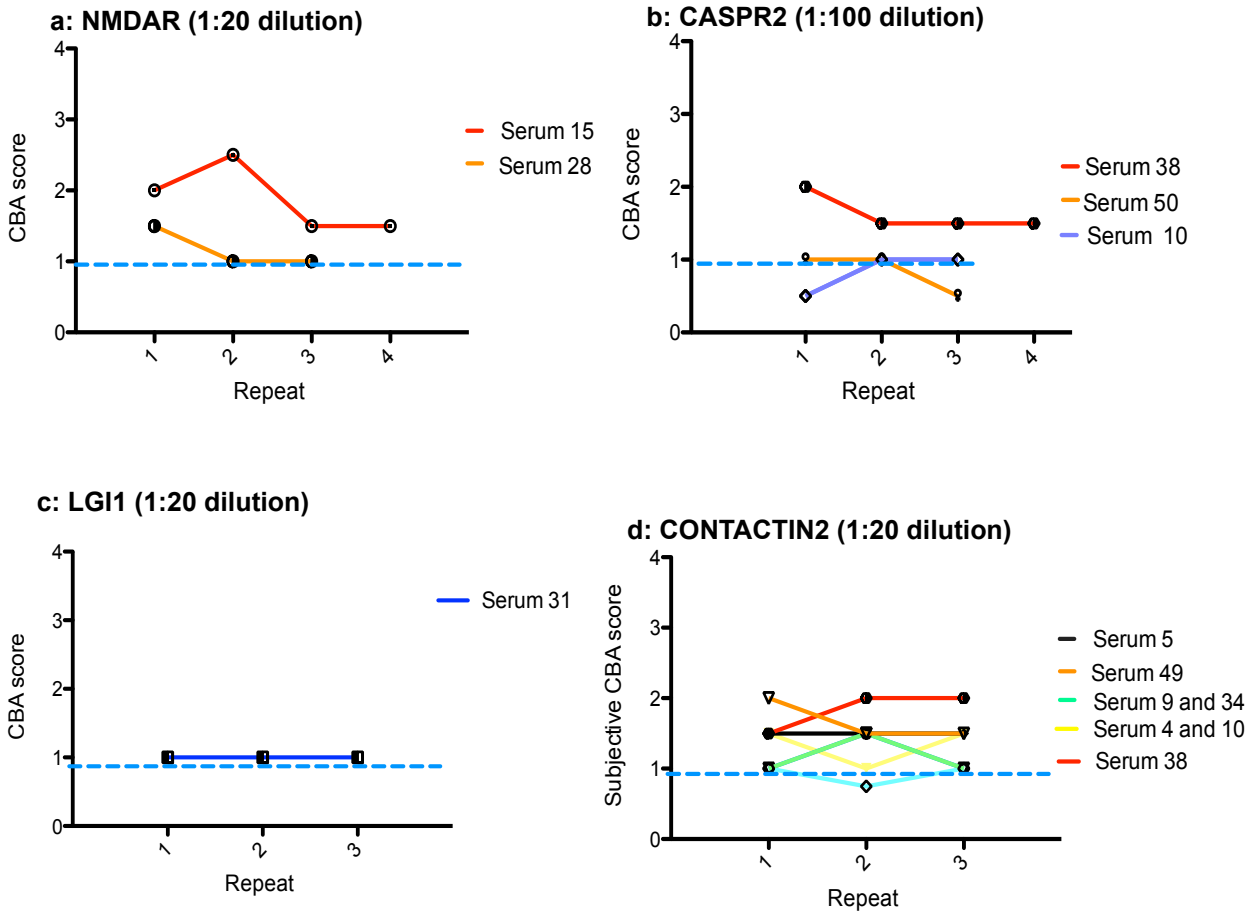


Figure 4.2: **Consistency of blinded CBA scoring on repeat assay**
All serum samples identified as positive in the CBA were repeated at least three times, and modal scores taken as the final score. Inter-assay scores often varied by 0.5 points although low-level positivity was repeatable. CONTACTIN2 was most frequently positive (n=7).

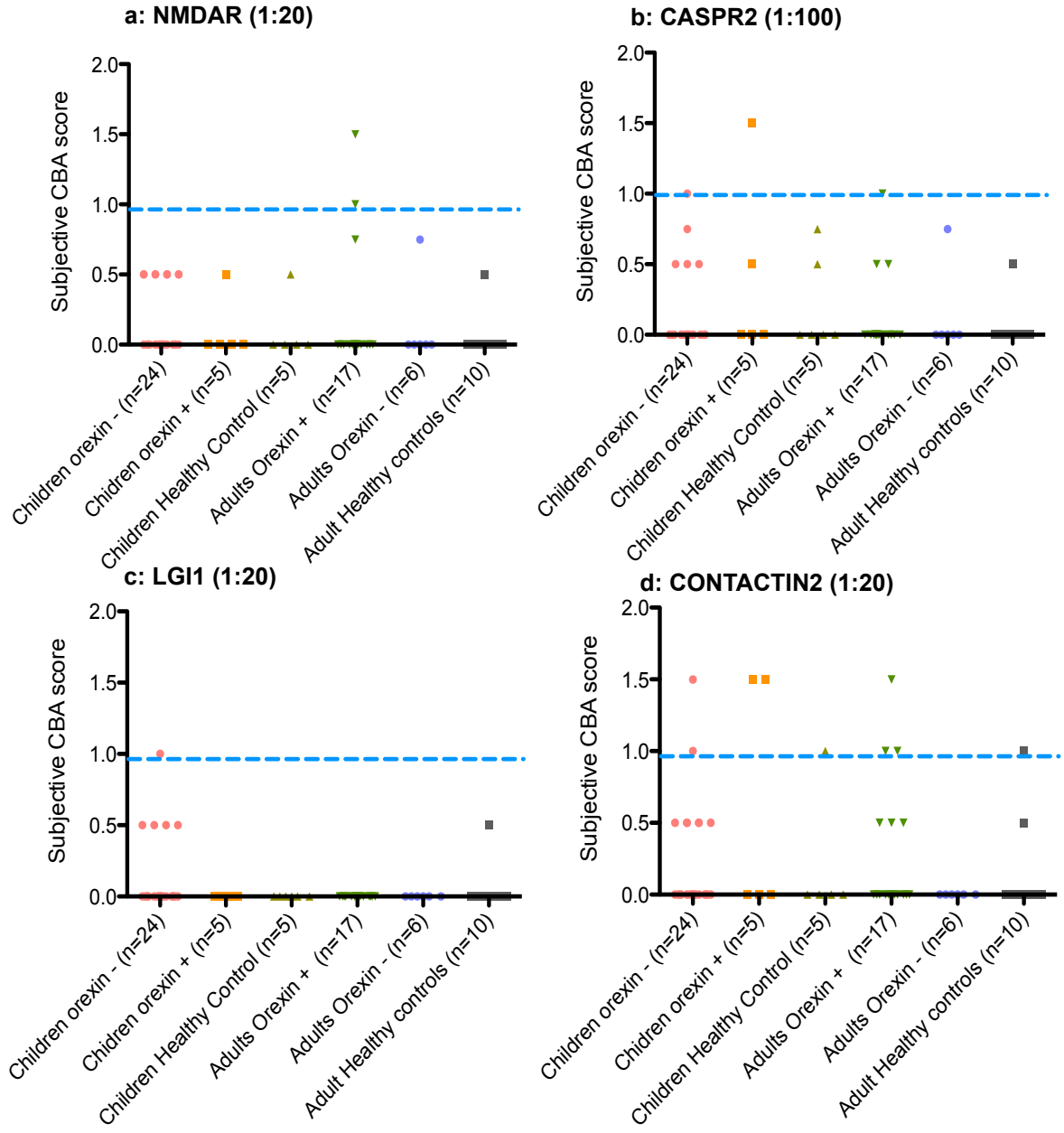


Figure 4.3: **Serum CBA scoring for four candidate antigens.** Higher levels of CBA positivity was not observed in orexin deficient children (**a-d**). Indeed, of the samples sent for orexin testing, positivity was more frequently observed in sera with normal levels of CSF orexin.
N.B. orexin - = orexin-deficient; orexin + = orexin-normal

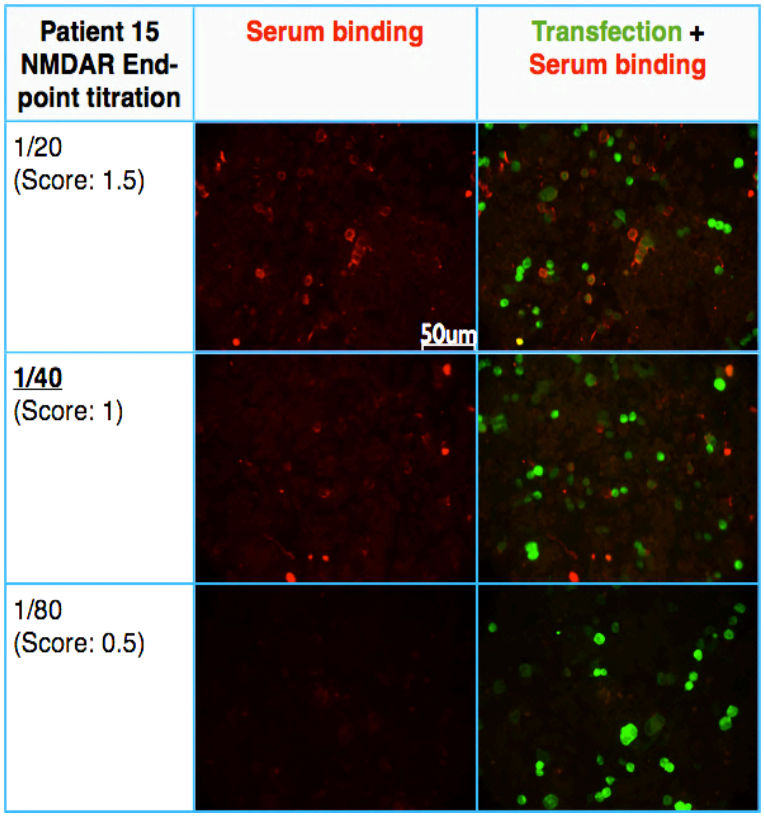


Figure 4.4: **Representative examples of CBA titrations** Titration of CBA positives confirmed low level positivity in 13 CBA positive sera

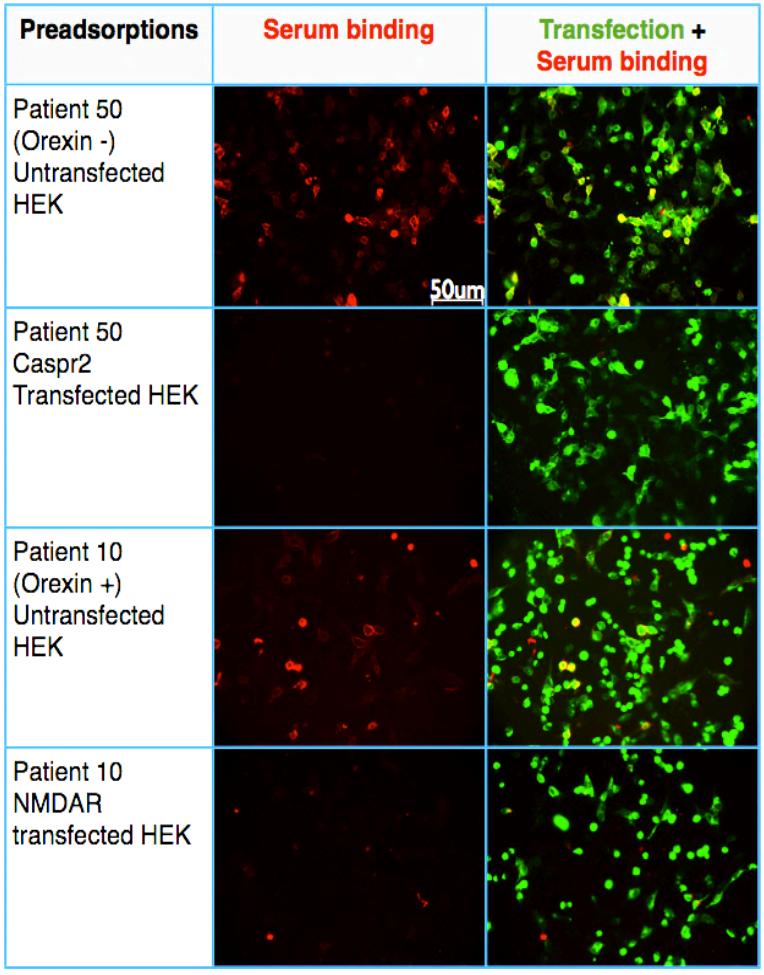


Figure 4.5: Representative examples of CBA preadsorptions Preadsorption of the 13 CBA positive sera against blank/ CASPR2 transfected HEK cells confirmed binding specificity

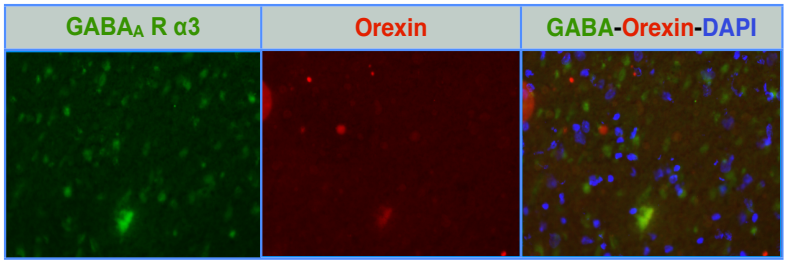


Figure 4.6: **Co-localisation of GABA$_A$Rα3 and orexin neurons by immunofluorescence (I.F.)** GABA$_A$Rα3 colocalised with orexin neurons. (see **chapter 5**)

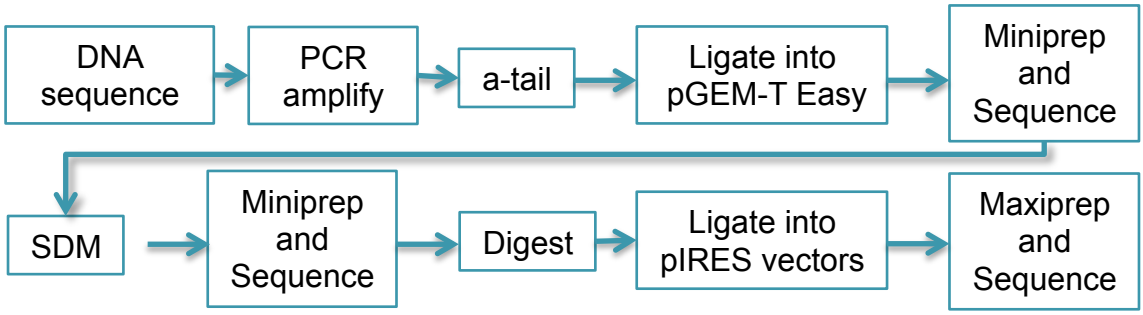
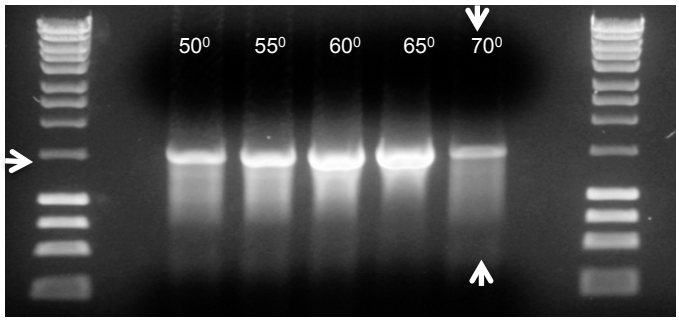


Figure 4.7: Flow diagram of molecular genetics steps undertaken with GABRA3
Abbreviations: PCR= Polymerase chain reaction, SDM= site-directed mutagenesis

a: PCR temperature gradient



b: XhoI1, BamH1 digest of pGEM-T Easy(GABA$_A$Rα3)

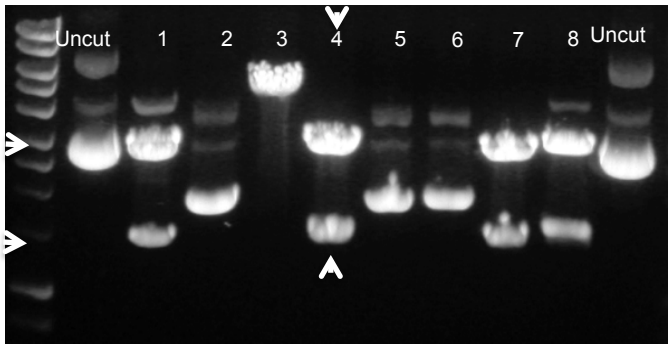


Figure 4.8: **Transferal of GABRA3 into pGEM-T Easy vector.** A cDNA clone was obtained for GABRA3 in the vector pBluescriptR. Following initial PCR sequencing, the insert was amplified by PCR at a specific at a range of annealing temperatures and then run on an 1% agarose gel (**a**). The 70^0 insert was selected for gel extraction and the 1.4kb band excised. After a-Tailing, the insert was ligated into pGEM-T Easy vector. Following transformation of JM109 cells and overnight incubation on ampicillin-supplemented plates, individual colonies were selected for amplification and miniprep. Following digestion with XhoI1 and BamH1, colony 4 was amplified by miniprep and sequenced as the 1.4kb insert was clearly seen to have been cut form the 3kb vector (**b**).
N.B. All temperatures are in degrees centigrade

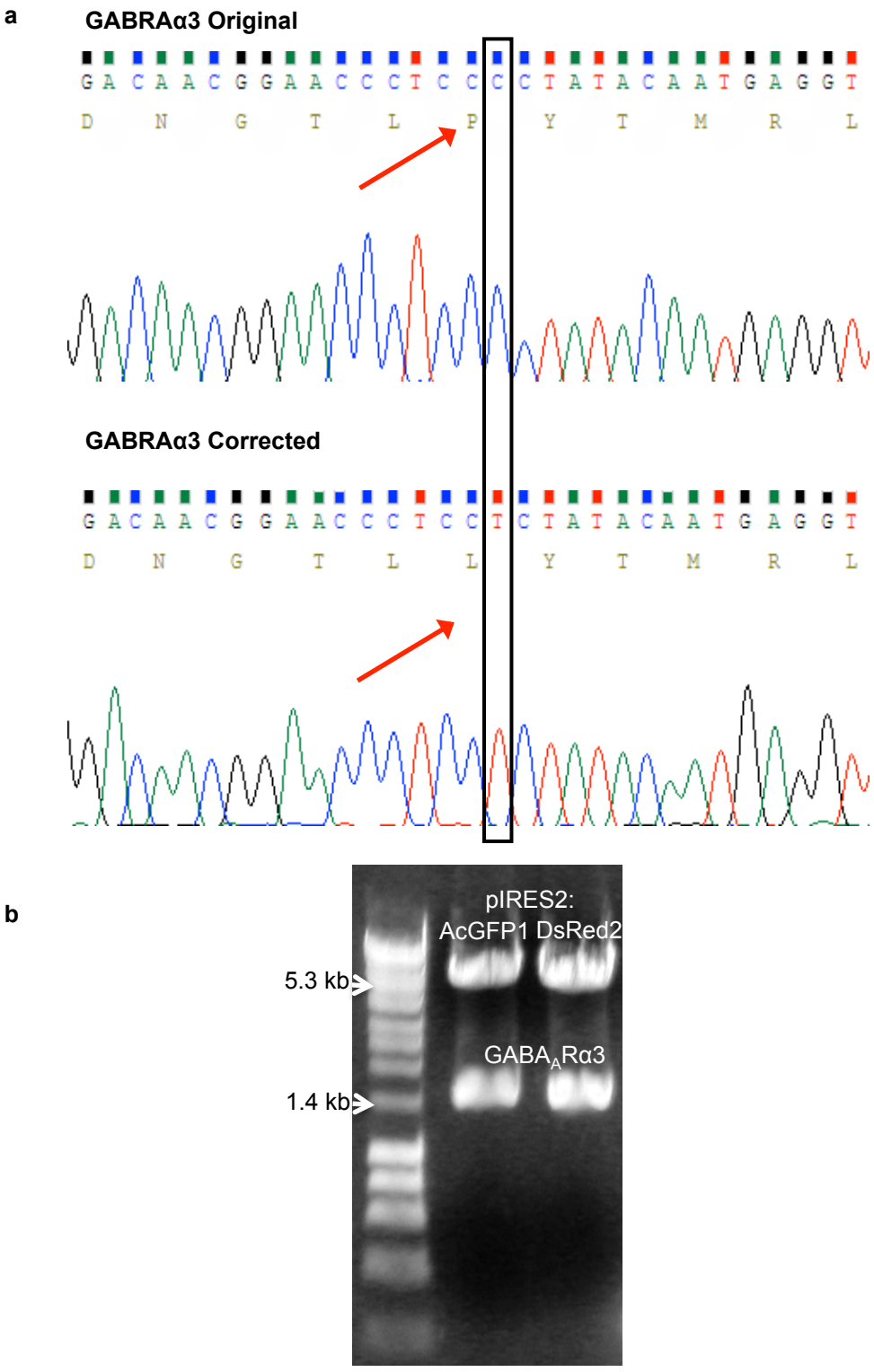


Figure 4.9: **Site directed mutagenesis of GABA$_A$Rα3 and ligation into pIRES mammalian vetors:** Site-directed mutagenesis using specifically-designed mutagenic primers was performed on the miniprepped DNA form colony 4 (**a**); c at position 778 was changed to t (boxed in black). This results in an amino acid change form proline to leucine (P180L: highlighted by red arrows). Following miniprep DNA amplification, the corrected insert was once again cut with XhoI1 and BamH1 and ligated into pIRES2-AcGFP1, a vector which can be expressed in mammalian cells (**b**). Following maxiprep and final PCR sequencing, the construct was used in conjugation with the other GABA$_A$ subunits to transfect HEK cells.

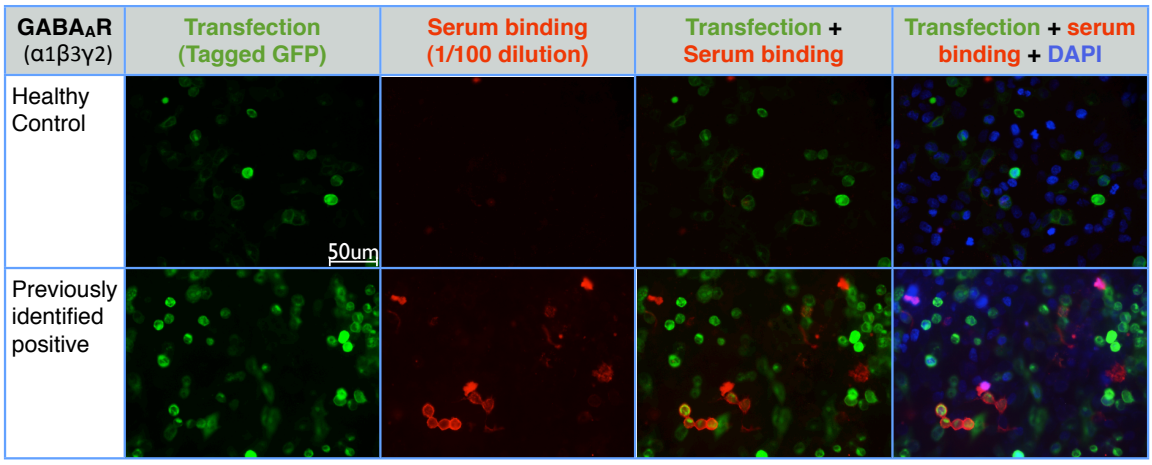


Figure 4.10: Positivity in one patient serum replicated on in-house GABA$_A$R constructs. A GABA$_A$R cell based assay had previously been used in the lab using α1β3γ2 subunits. DNA for these subunits was obtained and sequenced. Serum binding to γ2 subunit had been demonstrated in this patient's serum, a finding that was replicated here.

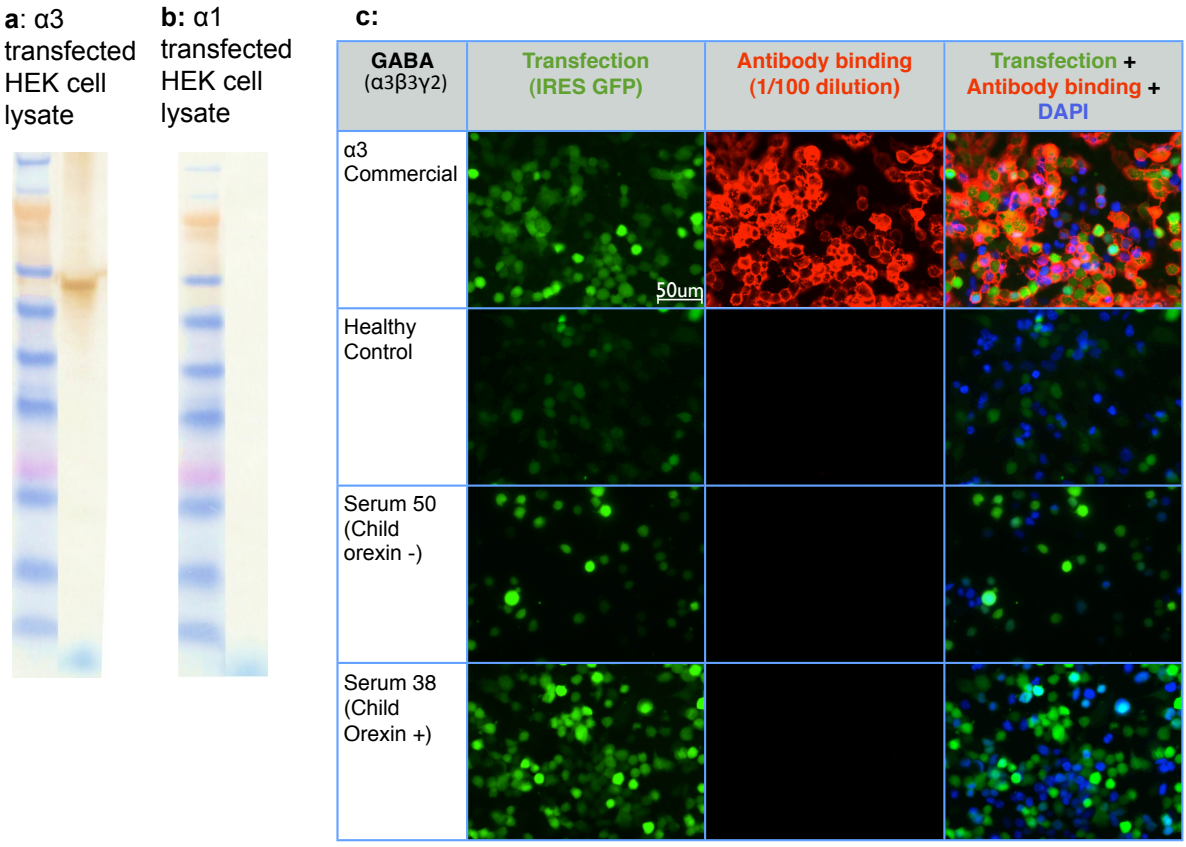
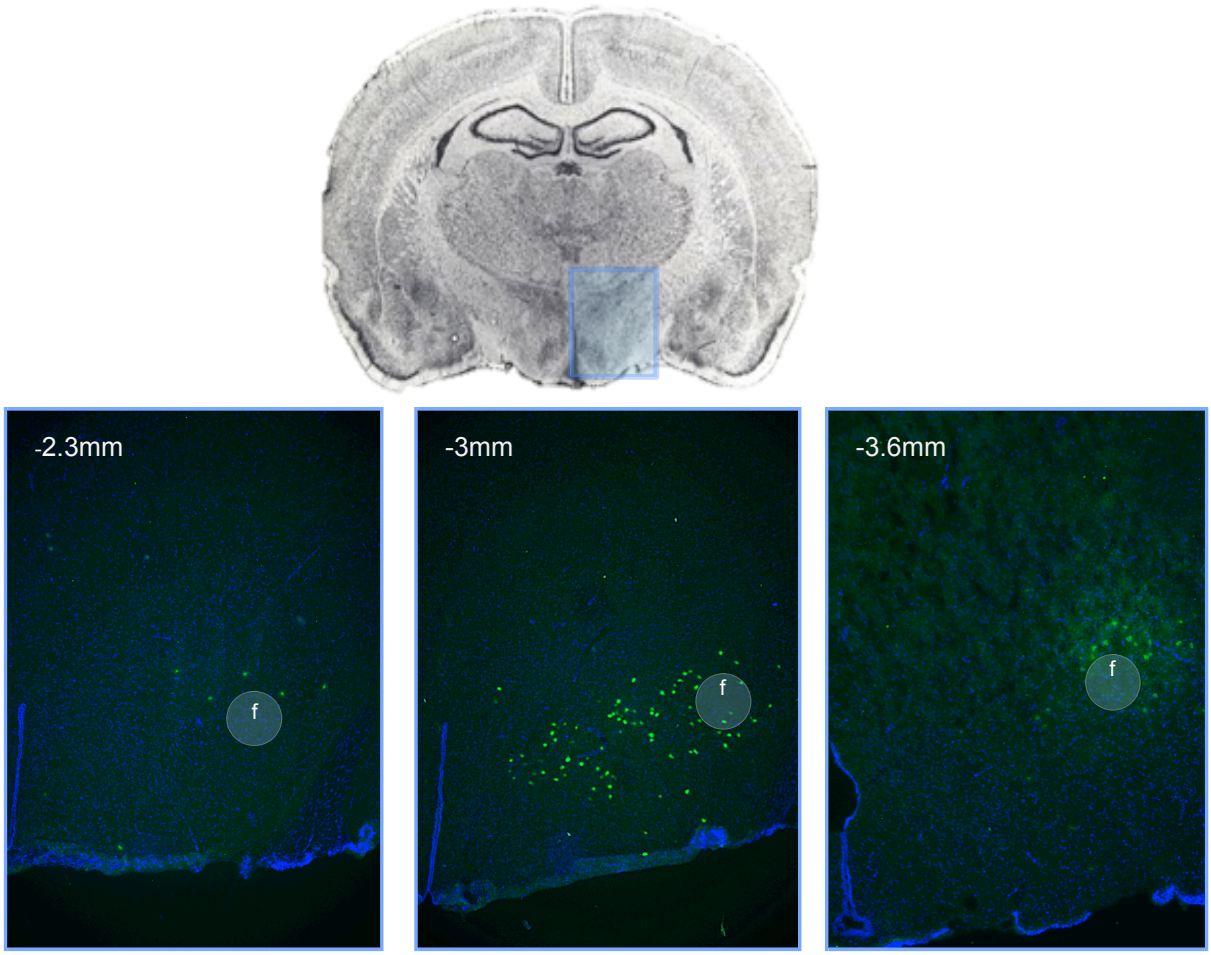


Figure 4.11: **GABA$_A$Rα3 successfully expressed on the surface of HEK cells. No positive sera found**

Western blot of lysate from HEK cells transfected with the α3-GFP, β3 and γ2 subunits showed a clear band at around 50-60kDa when incubated with a commercial anti-α3 antibody (**a**), whereas α1-GFP, β3 and γ2 transfected HEK cells showed no band (**b**). Cell surface expression of the α3 subunit was confirmed by strong commercial antibody binding on the CBA(**c**). However, after screening all serum samples on the α3 CBA, no positives whatsoever were found.

					Candidate antigen approaches						
					Serum CBA score					Serum RIA	
	Cohort No.	Sex	Age	CSF orexin A (pg/ml)	NMDAR 1:20	CASPR2 1:100	LGI1 1:20	CONTACTIN2 1:20	GABAa Rα3 1:20	VGKC	GAD
NDCN paired sera/CSF Orexin - Children	30	F	7	10	0	0	0	0	0	0	0
	18	F	8	10	0.5	0	0.5	0.5	0	0	0
	16	M	10	10	0	0	0	0	0	0	0
	33	M	10	10	0.5	0	0	0	0	0	0
	3	M	11	10	0	0	0	0.5	0	0	0
	53	F	13	10	0	0	0	0	0	0	0
	29	F	14	10	0	0.75	0	0	0	0	0
	57	M	16	10	-	-	-	-	0		0
NDCN paired sera/CSF Orexin + Children	13	F	5	363	0	0	0	0	0	0	0
	55	M	5	288	-	-	-	-	0	-	-
	56	M	8	217	-	-	-	-	0	-	-
	8	F	12	177	0	0	0	0	0	0	0
	54	M	12	265	-	-	-	-	0	-	-
	12	F	13	301	0	0.5	0	0	0	0	0
	5	F	14	262	0	0	0	1.5	0	0	0
Swedish post-vaccination narcolepsy-cataplexy	36	M	4	10	0	0	0	0	0	0	0
	39	F	8	10	0.5	0	0	0.5	0	0	0
	40	F	8	-	0	0	0	0	0	0	0
	37	M	9	52	0	0	0.5	0	0	0	0
	*38	F	9	290	0.5	1.5	0	1.5	0	0	0
	41	M	12	10	0	0	0.5	0	0	0	0
	42	M	12	10	0	0	0	0	0	0	0
	44	M	14	10	0	0.5	0	0.5	0	0	0
	35	F	15	10	0.5	0	0	0.5	0	0	0
	34	F	16	10	0	0	0	1	0	0	0
	43	M	16	10	0	0	0	0.5	0	0	0
Finnish post-vaccination narcolepsy-cataplexy	48	F	9	-	0.5	0	0	0	0	0	0
	47	F	12	-	0.5	0	0	0	0	0	0
	49	F	13	-	0	0	0	1.5	0	0	0
	51	M	17	10	0	0.5	0.5	0	0	0	0
	45	M	18	10	0.5	0.5	0	0	0	0	0
	50	F	18	10	0.5	1	0	0	0	156	0
	46	M	24	10	0	0	0	0	0	0	0
Children Heathy Control sera	HCC1			-	0	0.5	0	0	0	0	0
	HCC2			-	0.5	0	0	0	0	0	0
	HCC3			-	0	0	0	0	0	0	0
	HCC4			-	0	0	0.5	0	0	0	0
	HCC5			-	0	0	0	1	0	0	0

Table 4.2: Results summary for candidate antigen approaches on paediatric sera:
Within each grouping, sera are ordered by patient age. In a few of the NDCN sera, not all CBA experiments were possible because of insufficient serum volume denoted with a "-". Serum dilutions are stated alongside each of the candidate antigens.



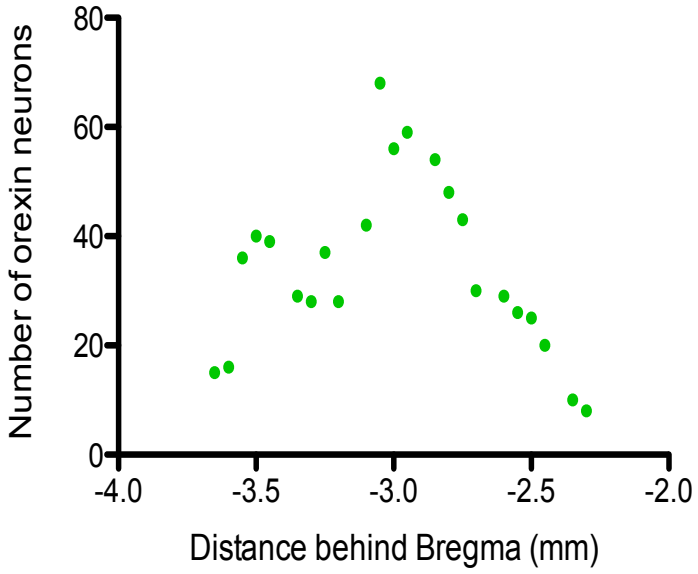


Figure 5.1: Coronal distribution of orexin and MCH neurons in the adult rat by I.F. Neurons were counted on one half of every 4th 12um rat coronal brain section. Orexin-a immunoreactive cell bodies were coronally distributed between -2.3 and 3.6mm relative to the bregma, and were generally clustered around the fornix (f).

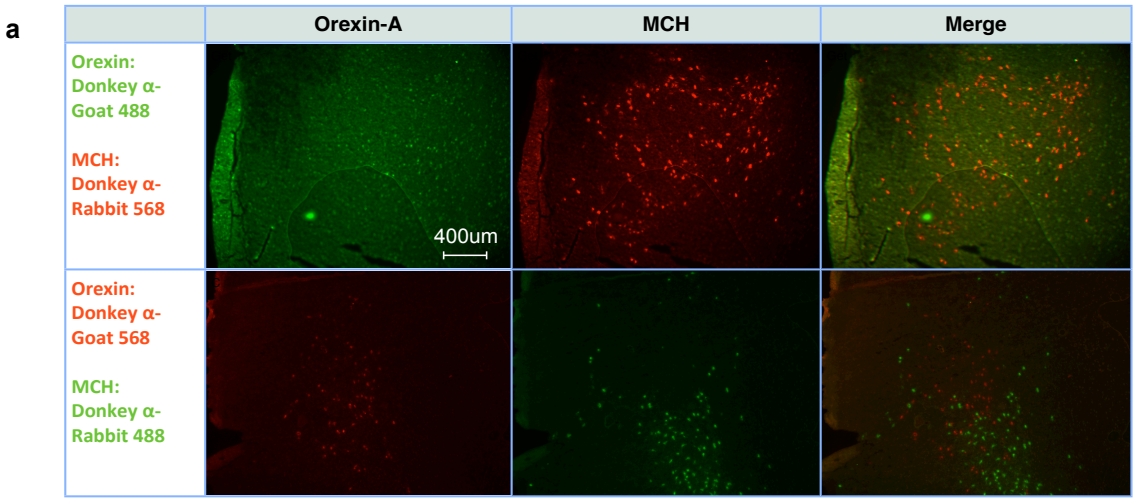


Figure 5.2: **Optimisation of orexin and MCH I.F. double-staining.** By immunflurescence (**a**), using a green secondary antibody for orexin in conjugation with red for MCH was found to give a high background. By reversing these colours, background was greatly reduced. Comparing I.F. to HRP-immunostaining, I.F. enabled clearer discrimination of orexin compared to MCH neurons, and was deemed to be more sensitive (**b**).

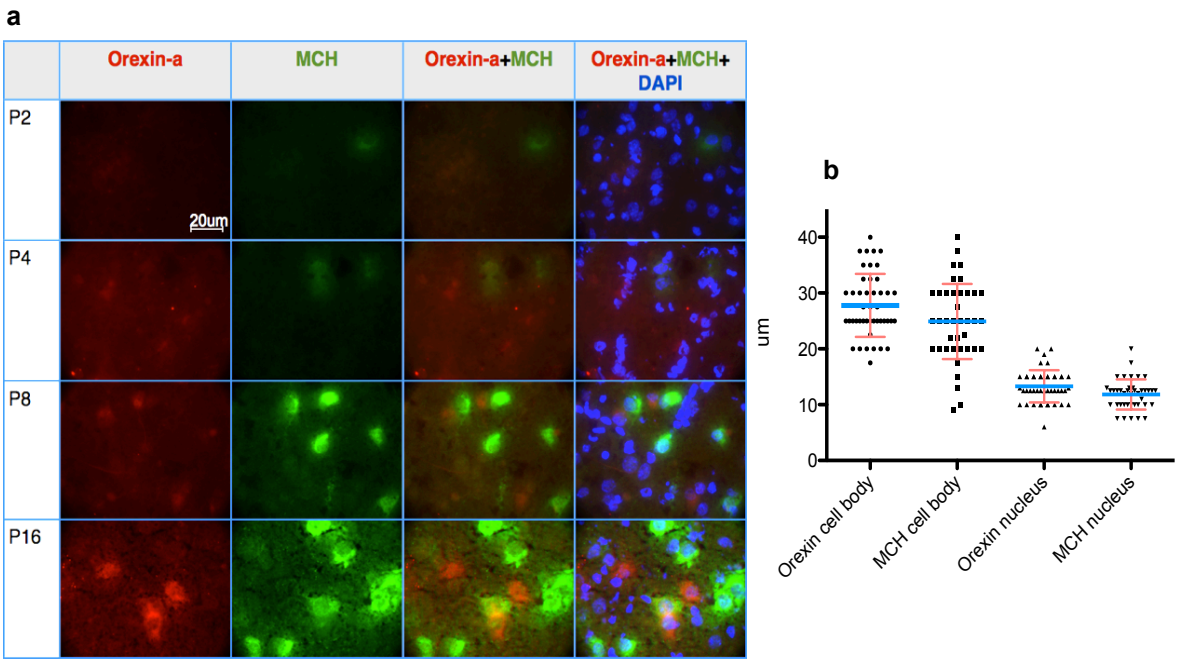


Figure 5.3: **Postnatal development of orexin and MCH and neuronal morphology in the adult rat.** For comparison, photos were taken immediately superior to the fornix. Whereas MCH neurons are clearly visable from P2, orexin neurons emerged by day 8 (**a**).
In the adult rat, a study of orexin and MCH neurons revealed similar morphology, with major cell diameters of around 25um (**b**).

a

Conditions tested:				
Fixation	Unfixed	3% PFA on the slide, 5 minutes	Immersion in acetone, 10 minutes	
Wash solution	PBS		PBST (1%)	
Blocking solution	10% Foetal Calf Serum		10% Normal Goat serum	
Serum Dilution	1/100	1/200	1/400	1/800
Serum incubation solution	10% Foetal calf serum in PBS	1% Foetal Calf Serum in PBS	10% Normal goat Serum in PBS	1% Normal goat Serum in PBS
Duration of serum incubation	1 hour at RTP		Overnight (16 hours) at 40C	
Choice of 2^{ry} antibody	Alexafluor 568 (red) diluted 1/1000 serum incubation solution		Alexafluor 488 (green) diluted 1/1000 in serum incubation solution	
Duration of 2^{ry} incubation	30 minutes		2 hours	

b

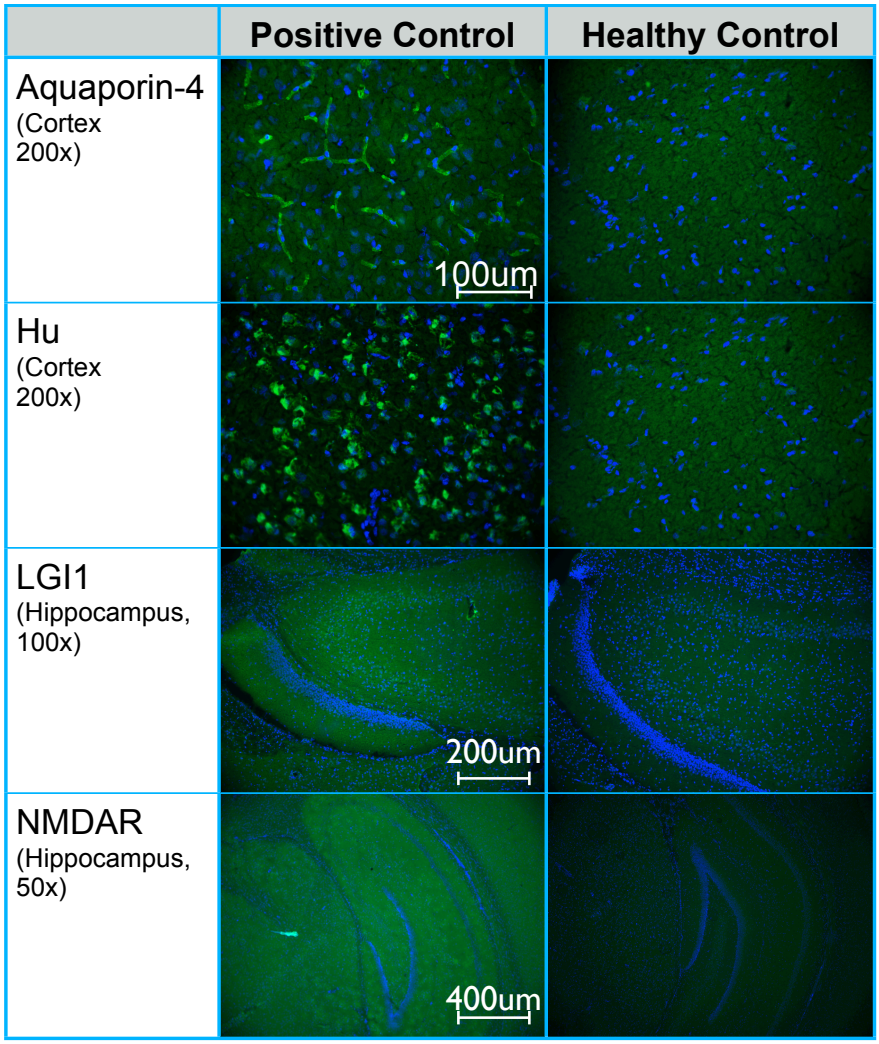


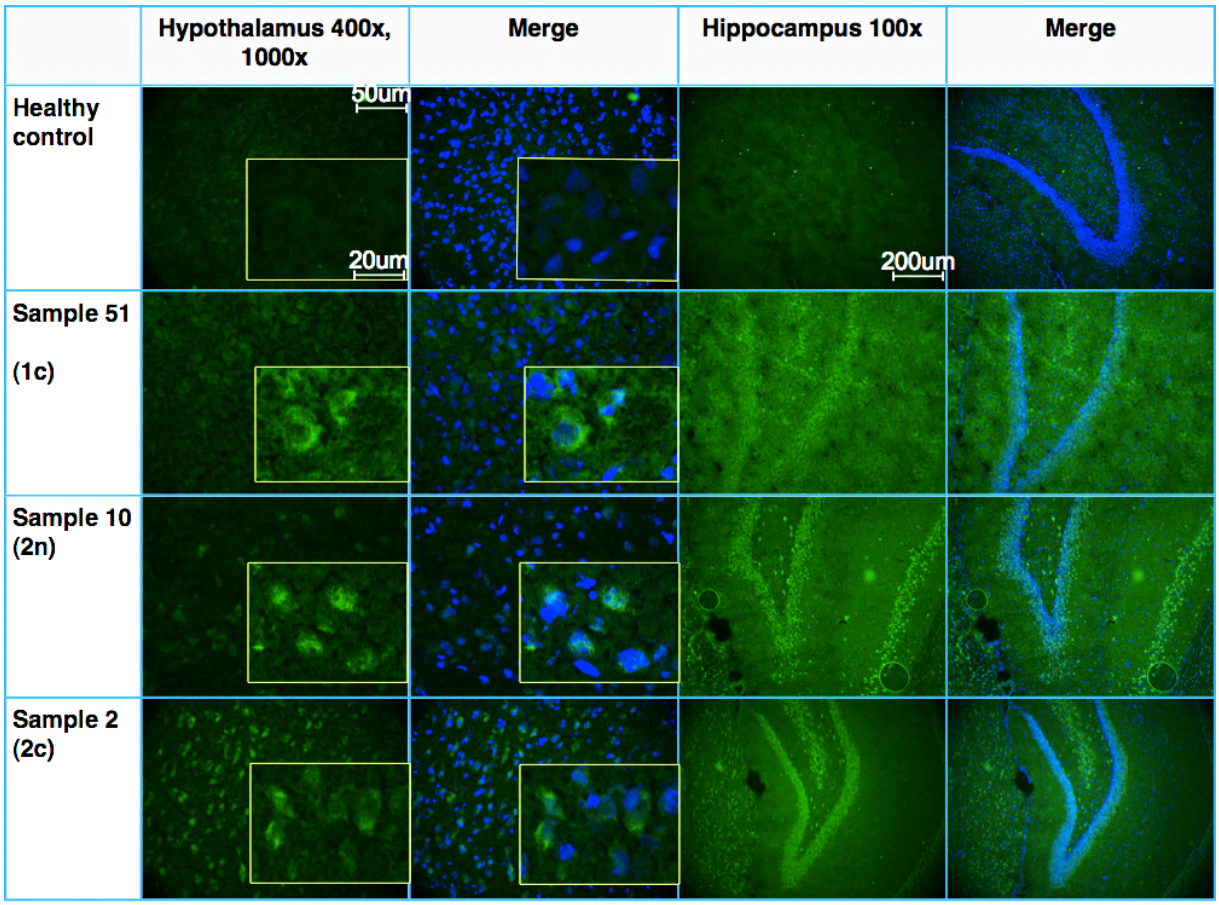
Figure 5.4: Optimisation of serum I.F. screen.
Prior to screening serum samples on coronal rat brain sections containing orexin neurons, a variety of test conditions were compared. The chosen optimal conditions are highlighted in blue (**a**).

These conditions best differentiated healthy controls from patterns observed with sera in other autoimmune diseases (**b**).

	Children			Adults				
	Orexin -	Orexin +	Healthy	Orexin -	Orexin +	Healthy	Pos. Controls	Total
Sera	24	8	5	6	17	10	5	n=75
CSF	29	9	0	17	15	0	0	n=70

Table 5.1: Samples tested by immunofluorescence immunohistochemistry (I.F.)
N.B. "Orexin –" = Orexin deficient. "Orexin +" = Orexin normal, Pos. Controls= positive controls

a



b:
Hippocampal binding in Serum 48:
33 year old female, CSF orexin normal, Contactin2 (1), Caspr2(1)

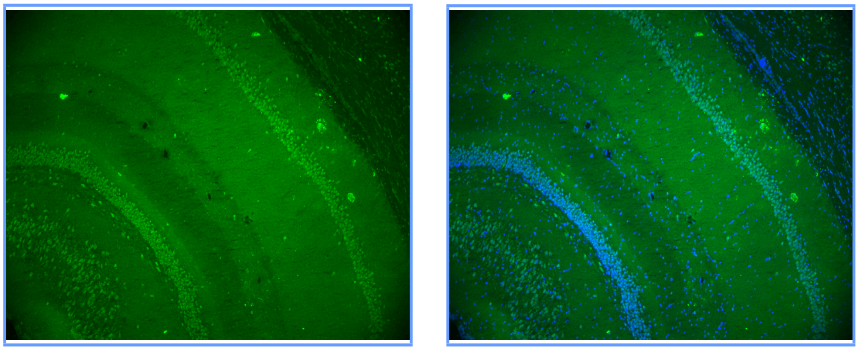


Figure 5.5: Initial serum I.F. screen: Sera were screened at 1:100 dilution. Observed binding was scored subjectively (0-3) and a note was made of cytosolic (c) or nuclear (n) patterns. Representative examples of binding observed is shown in (**a**). A hippocampal binding pattern was observed in Serum 48, most strong in the stratum moleculare and stratum radiatum (**b**).

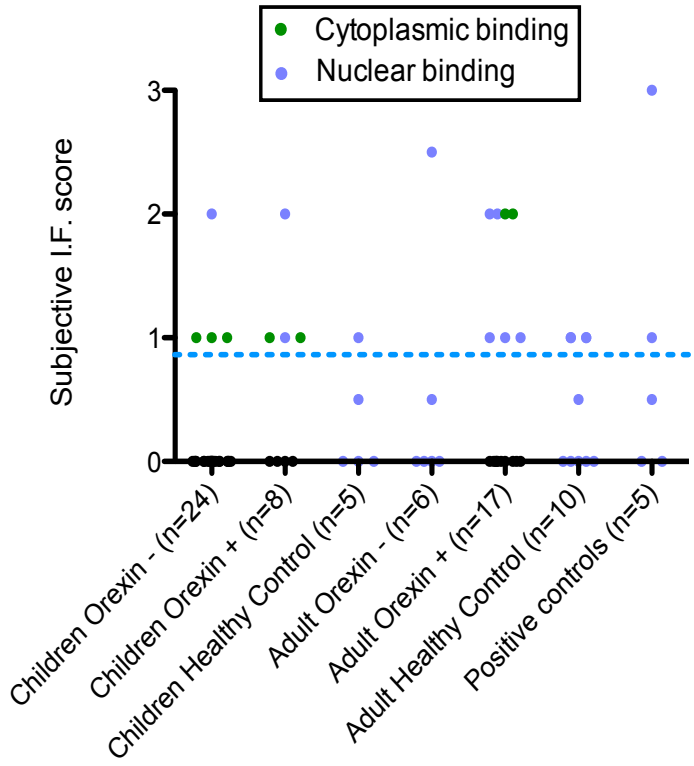


Figure 5.6: **Results of initial serum I.F. screen:** Serum binding in the hypothalamus did not appear to discriminate CSF orexin status or age. Although cytoplasmic patterns were observed in three orexin deficient children, similar binding was observed in other groups.

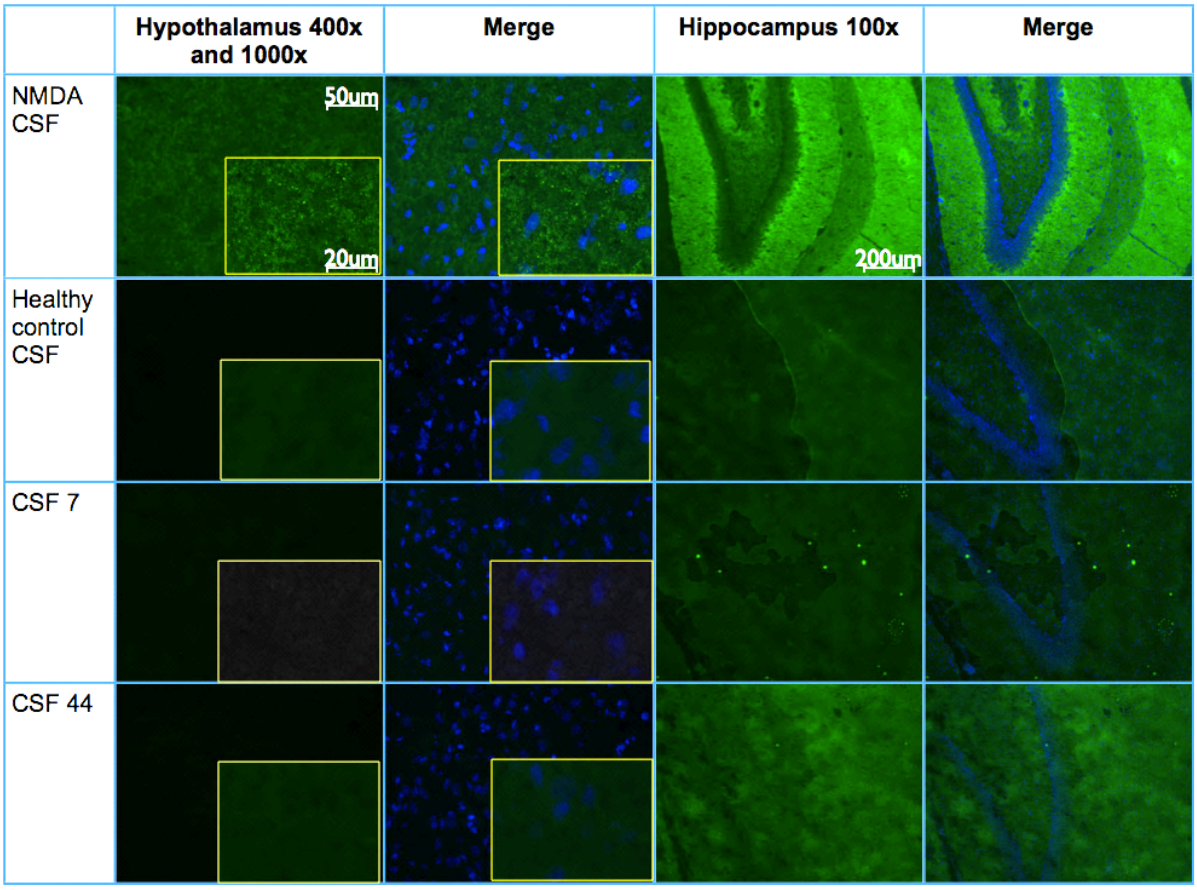


Figure 5.7: **Initial CSF I.F. screen**: 70 patient CSF's were screened by IF at 1:1 dilution. Clear neurophil binding of the hippocampal inner molecular layer was observed with the CSF of an NMDA positive control, but no binding was observed with narcoleptic patient CSF's.

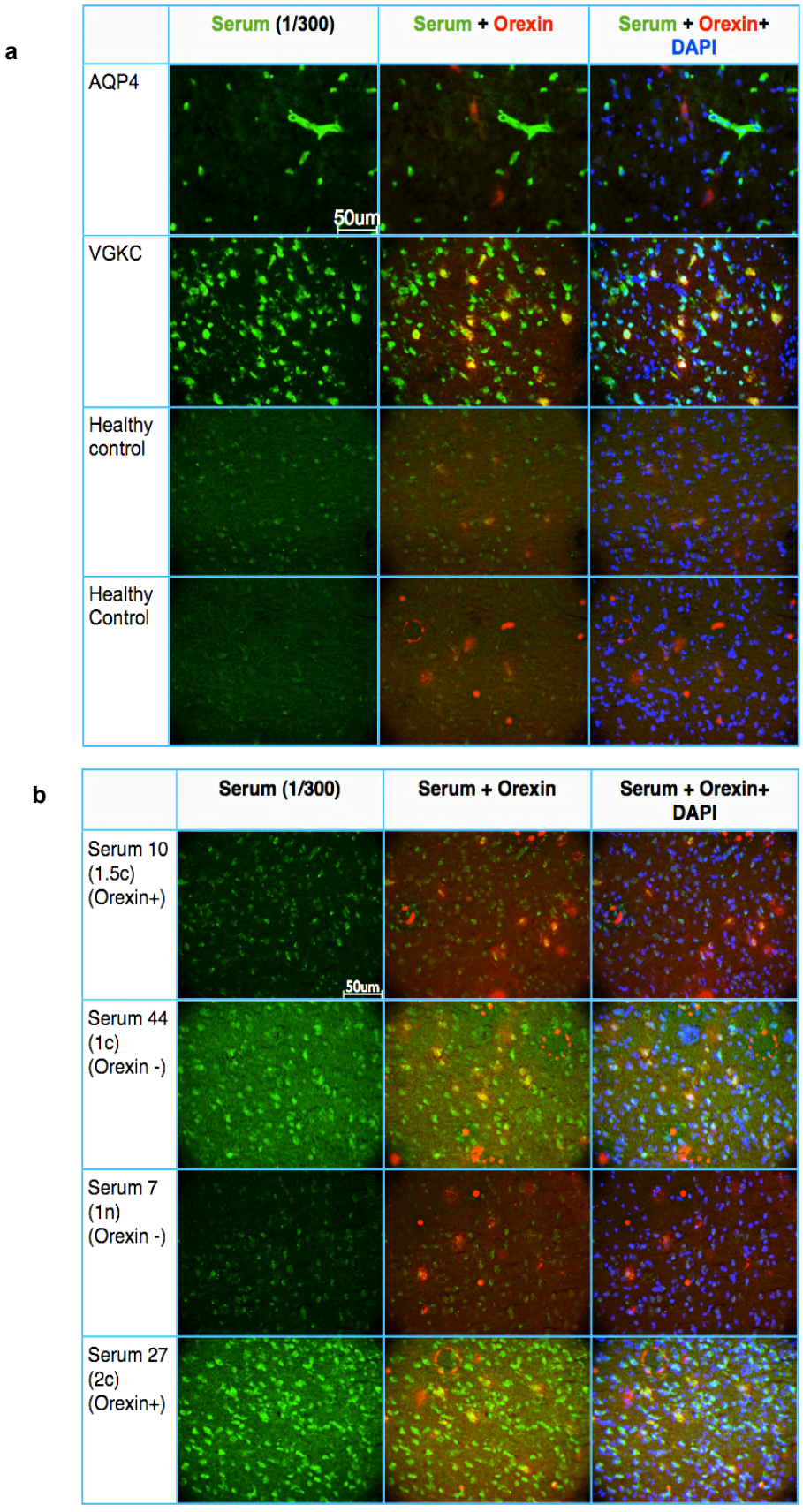
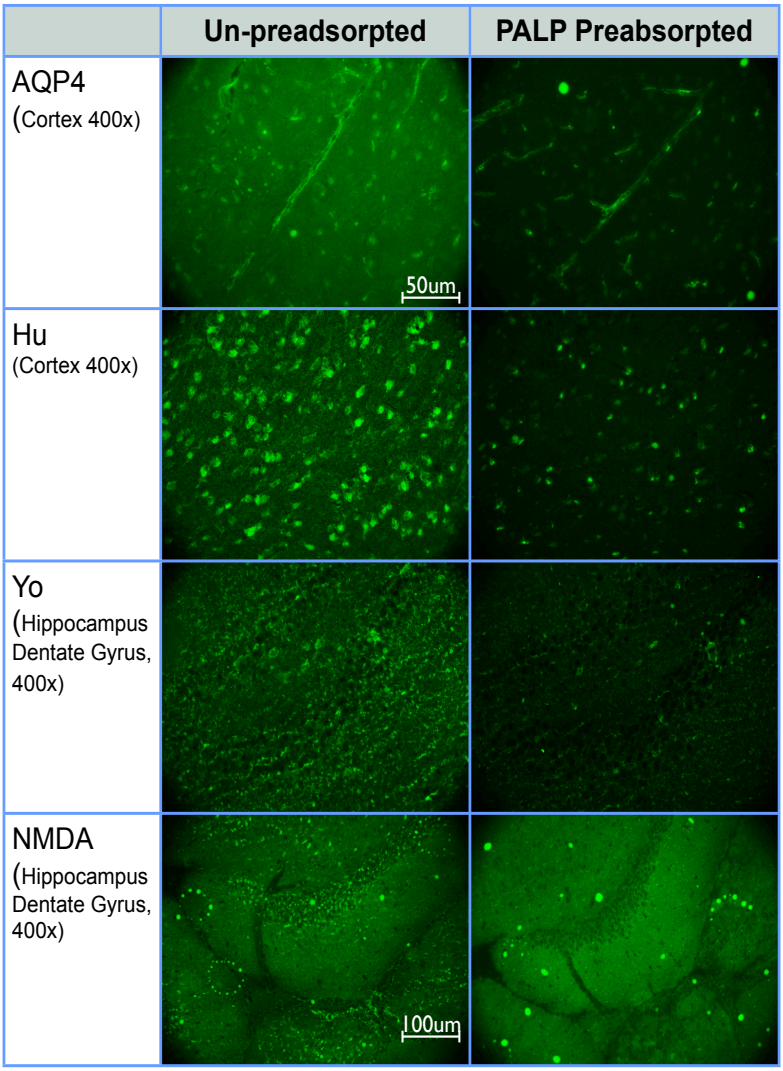


Figure 5.8: **Serum-orexin colocalisation experiments**: Co-localisation of serum binding and orexin immunoreactivity was first optimised using autoimmune disease and healthy control sera (**a**). Patient serum identified as reactive on the initial IF screen were selected for co-localisation experiments (representative examples shown in **b**).

a



b

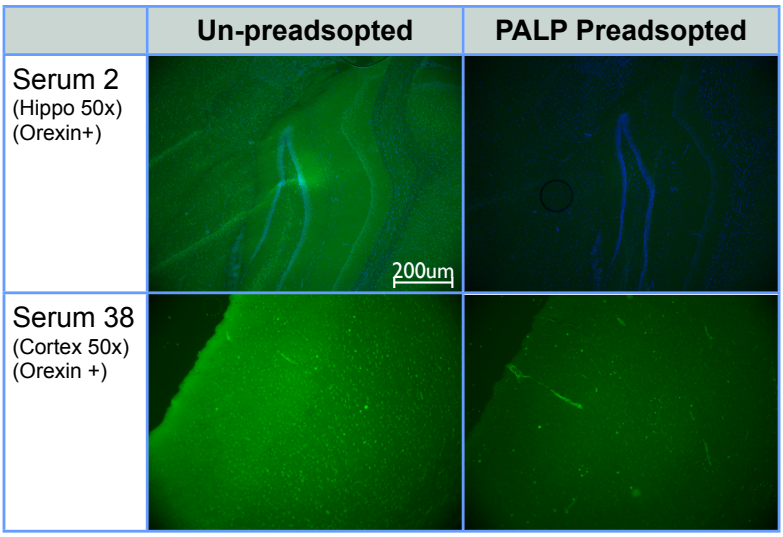


Figure 5.9: **Optimisation of pigeon acetone liver powder (PALP) preadsorption:** Preadsorbsion with PALP apparently reduced non-specific serum binding. Specific binding patterns observed in positive control sera were maintained, whilst background was reduced (**a**). With previously immunoreactive narcolepsy sera, apparent binding was greatly reduced in most cases, although some patterns became clearer (**b**). In the above example, blood vessel binding in serum 38 only became visible after preadsorbsion, when surrounding background was reduced.

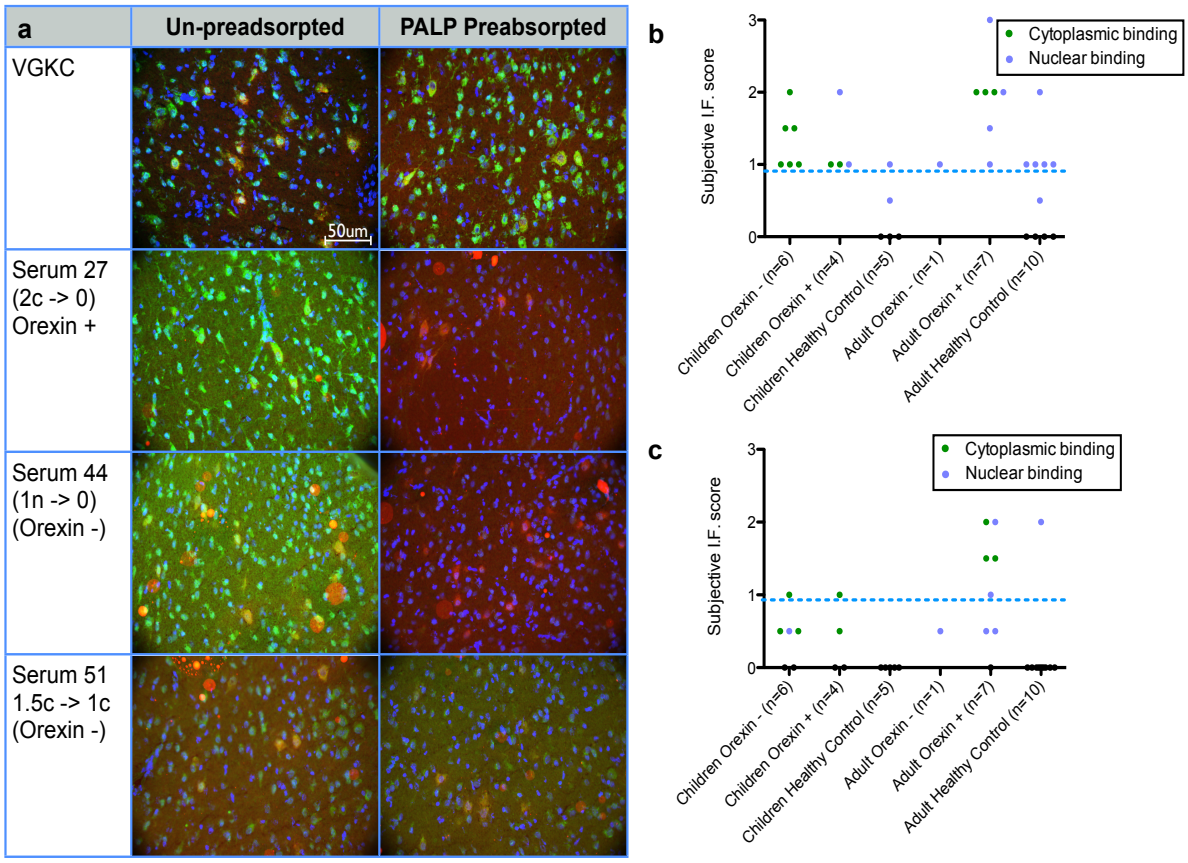


Figure 5.10: **Colocalisation of serum with orexin neurons pre and post PALP preadsorbsion:** Representative examples of serum binding patterns to orexin neurons are shown in (**a**)(Green= serum binding, Red= orexin neurons, Blue= DAPI)
Subjective scores of serum/orexin neuron co-localisation on un-preadsorbed and PALP preadsorbed sera are shown in (**b**) and (**c**) respectively. After preadsorbsion, orexin normal adults scored most highly.

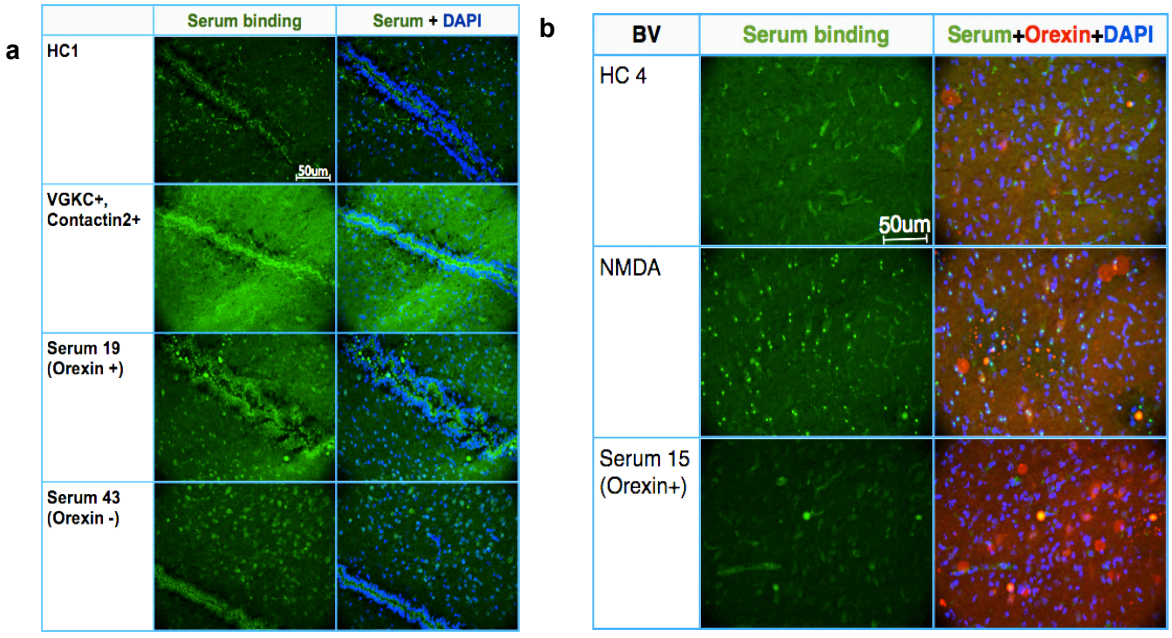
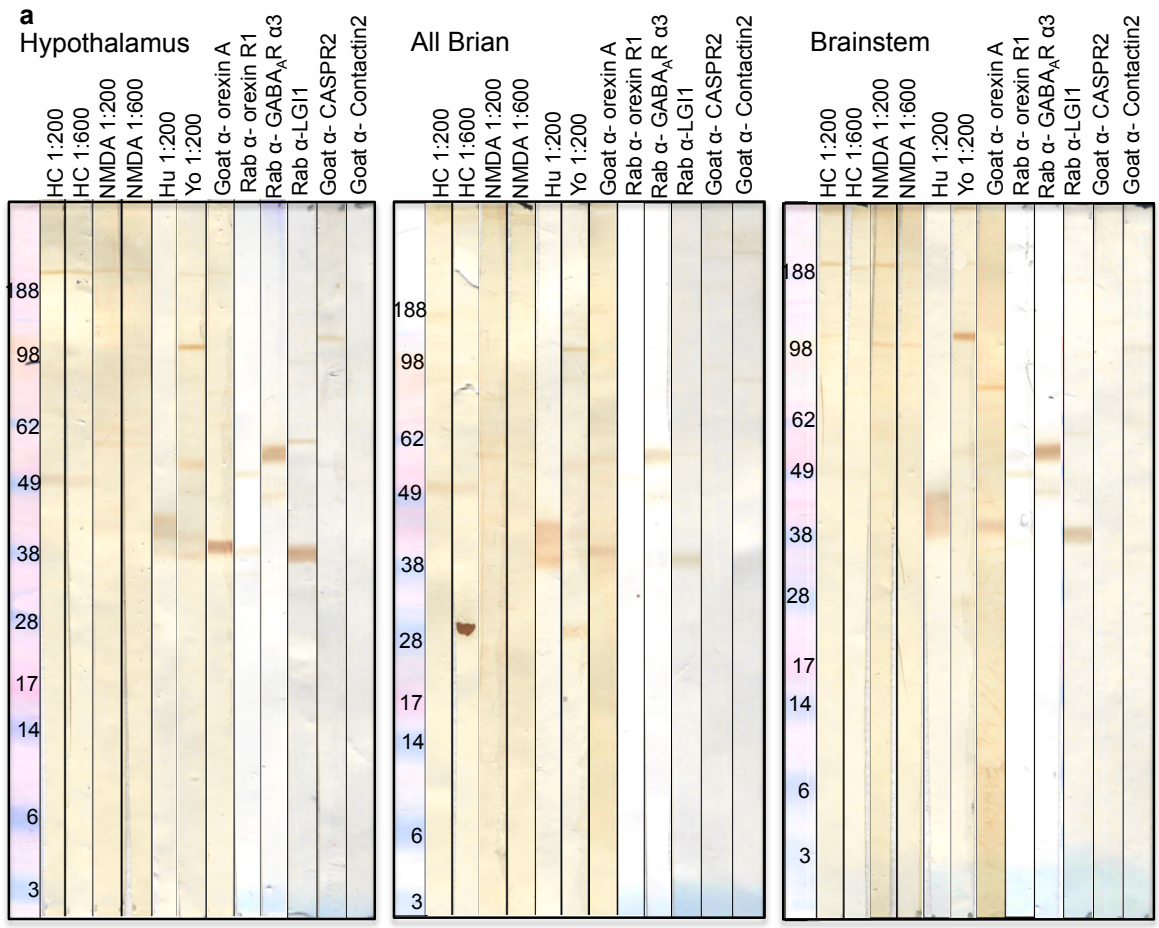


Figure 5.11: **Ventricular and blood vessel patterns observed after PALP preadsorbsion**, Following preadsorbsion, patterns outside of the hypothalamus became more apparent in some cases. Binding to the apical aspect ependymal cells in the 3rd ventricle (3rdV) was observed in number of samples (**a**). Additionally, binding to blood vessels (BV) was observed (**b**). However, these patterns were observed in all groups tested including healthy controls.



b

Autoantigen	Expected Band (kDa)	Observed Bands (kDa)
NMDAR serum	116, 180	42, 60, 100, 200
Hu serum	40	35-45
Yo serum	34, 52, 62	35, 40, 55, 100
Caspr2 commercial	180	100 (Hypothalamus only)
Lgi1 commercial	60 and 70	40 and 55
Contactin2 commercial	150	90 (All brain and Brainstem)
GABA A R a3 commercial	50	50-55
Tribbles-2 commercial	37	None observed

c

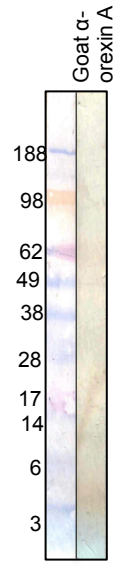


Figure 5.13: **Optimisation of brain extract western blots**:
Blots of each brain region are displayed in (**a**). Conditions applied to each strip are written vertically above the relevant strip. Expected/observed bands for positive control sera/ commercial antibodies are summarised in the adjacent table (**b**).
An orexin peptide blot probed with an anti-orexin commercial antibody confirms the presence of the 3kDa peptide (**c**)

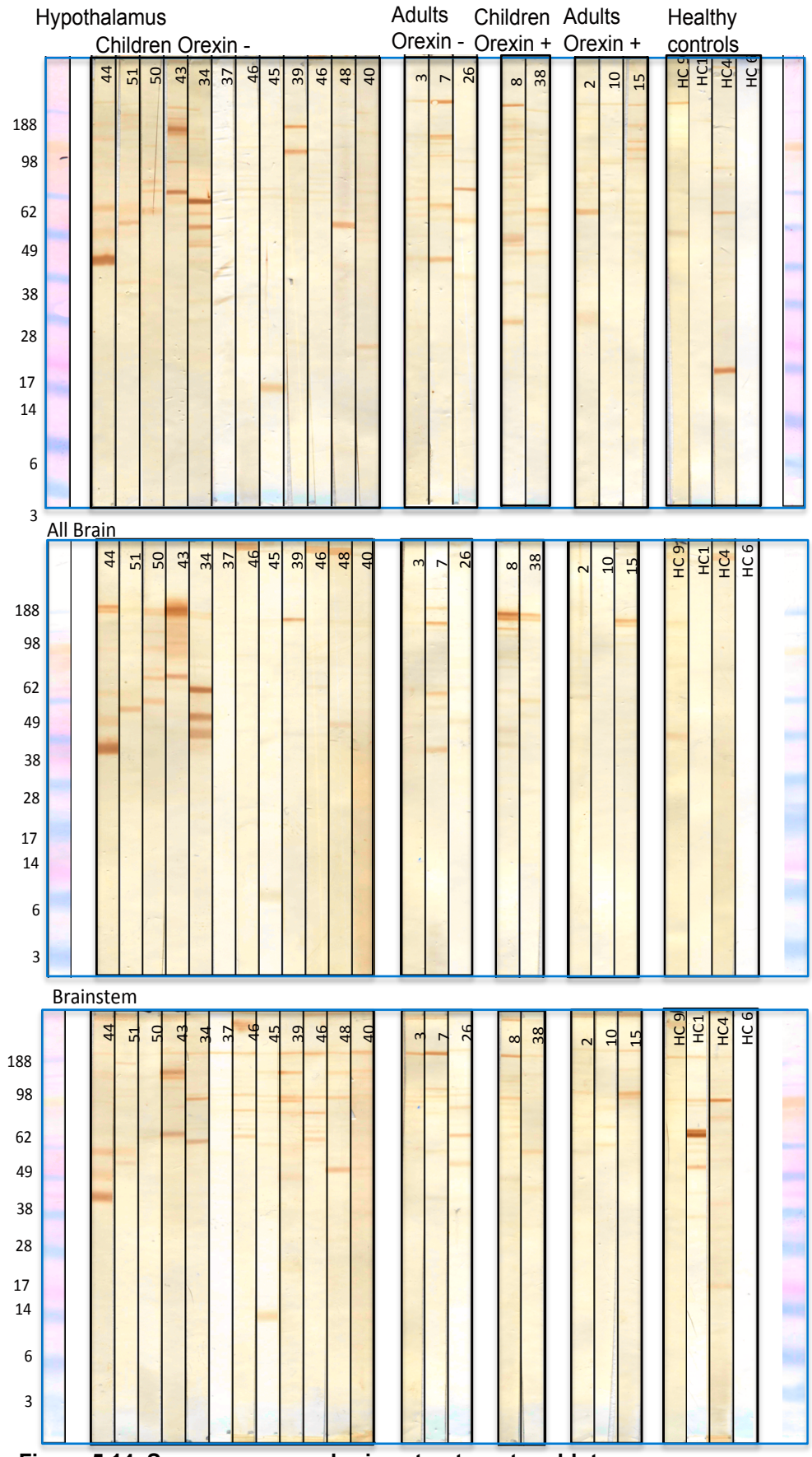


Figure 5.14: **Serum screen on brain extract western blots**:
N.B. The order of strips has been rearranged into appropriate groupings.
Approximate molecular weights (kDa) of dye markers are included in every blots along the left hand side.
The serum sample used to probe each strip is written vertically at the top.

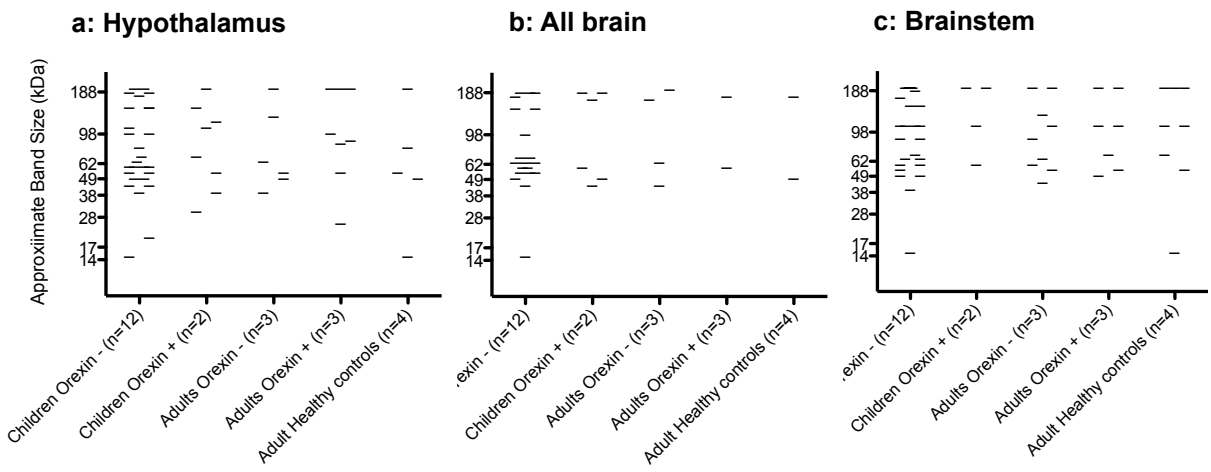
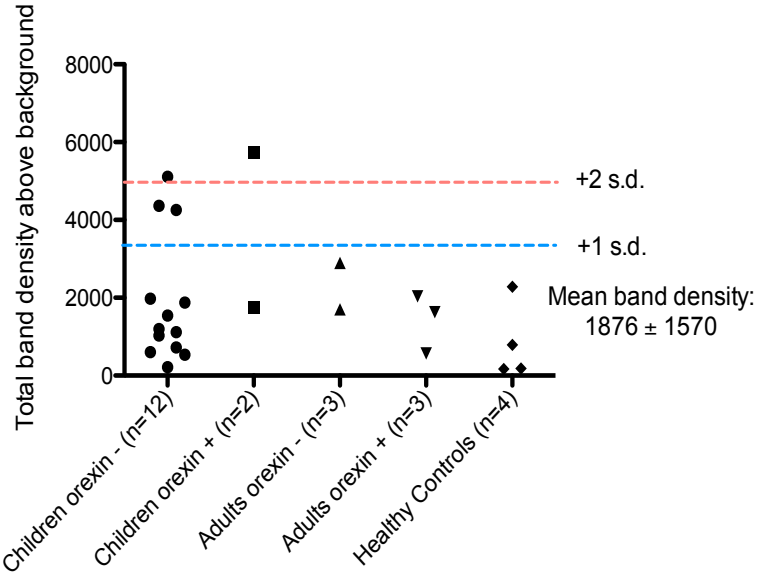


Figure 5.15: **Distribution of serum bands in brain extract western blots:**
A log $_2$ scale was used to plot molecular weight, approximating the gradations of the dyed markers. No clear pattern of bands was discernable in any particular group.



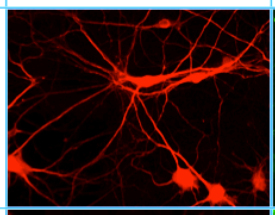
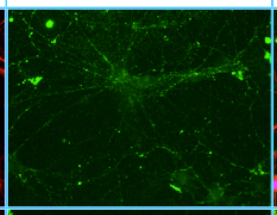
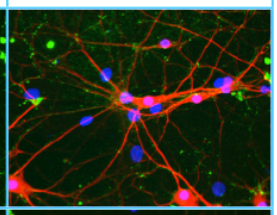
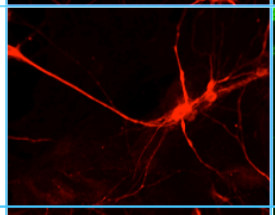
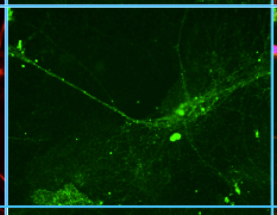
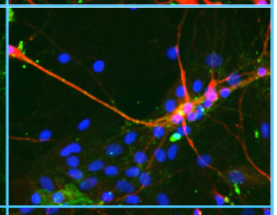
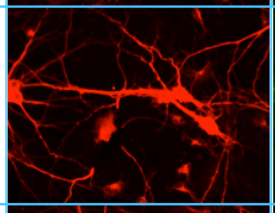
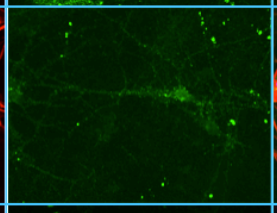
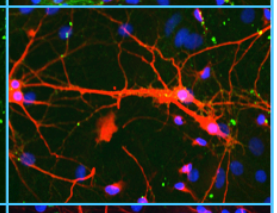
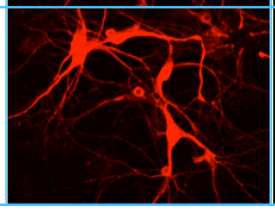
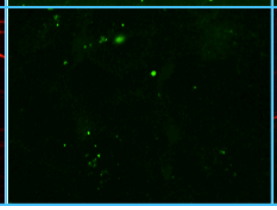
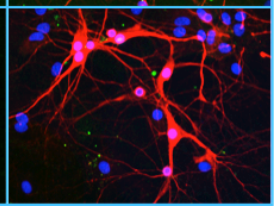
Serum	Cohort	Serum IgG	CBA	IHC
44	Child orexin -	12.6 mg/ml	Caspr2: 0.5, Contactin2: 0.5	2c
43	Child orexin -	2.8 mg/ml	Conactin2: 0.5	1.5c
34	Child orexin -	3.2 mg/ml	Contactin2: 1	0
8	Child orexin +	4.2 mg/ml	0	2n

Figure 5.16: Analysis of total band densities in the hypothalamus:
Four sera from children had total band densities greater than the population mean + 1s.d. (blue dotted line) and 2 had densities more than mean +2sd (red dotted line).
The table summarises other laboratory findings of these patients. Total IgG did not seem to be a determinant of high total band density. However, all of these samples had previous reactivity on CBA and/or IHC. Samples with densities more than 2s.d above the population mean are highlighted in bold.

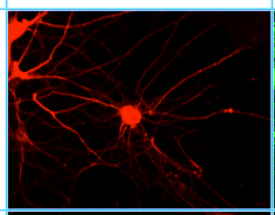
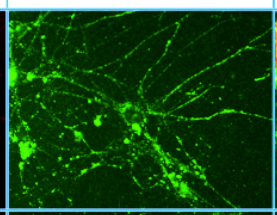
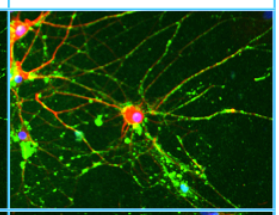
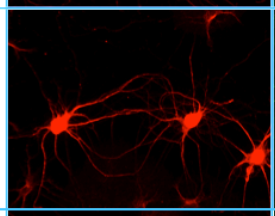
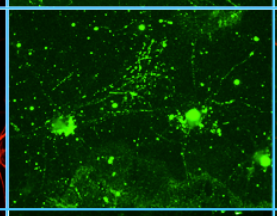
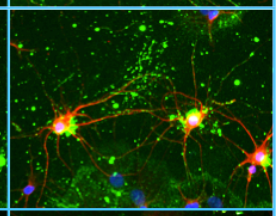
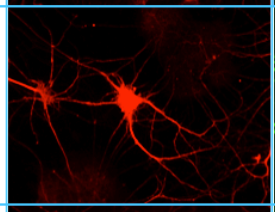
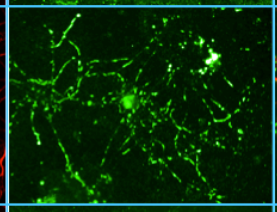
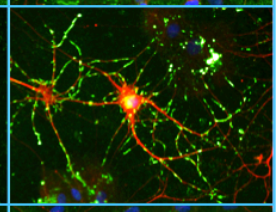
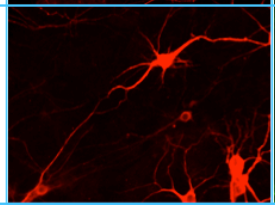
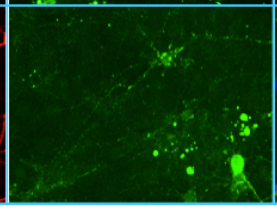
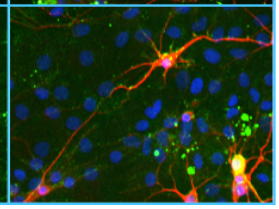
		Clinical		Biomarkers	Screening approaches			
				CSF	Serum I.F.			Western blot total band density (hypo) (pixels/inch2)
	Cohort No.	Sex	Age	Orexin A	Initial I.F., 1:100 hippo	Orexin co-localisation 1:300	PALP Preabsorbed orexin co-localisation 1:300	
NDCN paired sera/CSF Orexin - Children	30	F	7	10	0			-
	18	F	8	10	0			-
	16	M	10	10	0			-
	33	M	10	10	0			-
	3	M	11	10	2n	1c	0	1027.6
	53	F	13	10	0			-
	29	F	14	10	1c			-
	57	M	16	10	0			-
NDCN paired sera/CSF Orexin + Children	13	F	5	363	1c	-		-
	55	M	5	288	0			-
	56	M	8	217	0			-
	8	F	12	177	2n	2n	0.5n	5746.1
	54	M	12	265	0			-
	12	F	13	301	0			-
	5	F	14	262	1n	1n	0	-
Swedish post-vaccination narcolepsy-cataplexy	36	M	4	10	0			-
	39	F	8	10	0			1879.6
	40	F	8	-	0			603.4
	37	M	9	52	0			723.5
	*38	F	9	290	1c	0	1c	1742.3
	41	M	12	10	0			-
	42	M	12	10	0			-
	44	M	14	10	0	2c	0.5c	5112.6
	35	F	15	10	0			-
	34	F	16	10	0			4257.5
	43	M	16	10	0	1.5c	0.5c	4364.4
Finnish post-vaccination narcolepsy-cataplexy	48	F	9	-	0			1119.3
	47	F	12	-	0			-
	49	F	13	-	0			-
	51	M	17	10	1c	1c	1c	1974.6
	45	M	18	10	0			1545.7
	50	F	18	10	1c	1.5c	0.5c	1197.4
	46	M	24	10	0			534.2
Children Heathy Control sera	HCC1			-	0	0	0	-
	HCC2			-	0.5n	0.5n	0	-
	HCC3			-	0	0	0	-
	HCC4			-	1n	1n	0	-
	HCC5			-	0	0	0	-

Table 5.2: Results summary for screening approaches on paediatric sera

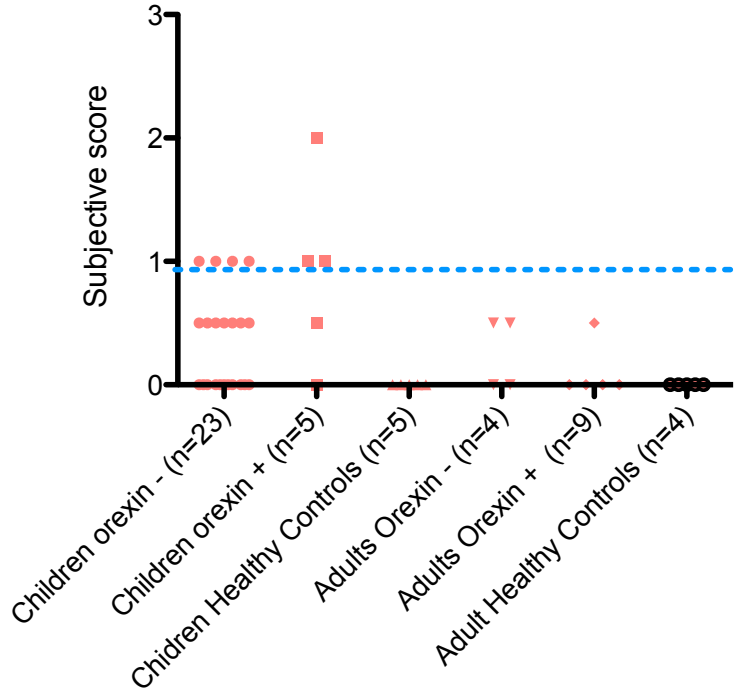
Figure 6.1 a: Positive control binding

Hippocampal neuronal cultures 1/100	MAP2	Serum Binding	MAP2 + Serum Binding +DAPI
CASPR2			
LGI1			
NMDAR			
Healthy Control			

b: Paediatric serum binding

Hippocampal neuronal cultures 1/100	MAP2	Serum Binding	MAP2 + Serum Binding +DAPI
Serum 5 (Orexin + child TAG1 1.5)			
Serum 38 Orexin + child (Caspr2: 1.5 Tag1: 1.5)			
Serum 46 (Orexin - child)			
Serum 50 (Orexin - child Caspr2: 1)			

c: Subjective scores of hippocampal cultures



d: Summary of prior findings of sera that bound to hippocampal neurons

Sample	Age	Sex	Orexin status	Prior findings	Hippocampal cultures score
3	12	M	-	I.F.: 2n on initial screen, 1c orexin-colocalisation	1
39	8	F	-	None	1
35	15	F	-	None	1
46	14	M	-	None	1
48	9	F	?	None	1
5	15	F	+	CBA: 1.5 Contactin2; I.F.: 1n initial screen, 1n orexin co-localisation	1
8	12	F	+	I.F.: 2n on initial screen and orexin co-localisation, WB: strongest total binding to hypothalamic brain extract	1
38	9	F	+	CBA: Contactin 2 and Caspr2: 1.5; I.F.: 1c on initial screen and after PALP preabsorbsion	2

Figure 6.1: **Serum screening on hippocampal neuronal cultures**
After confirming that the neuronal cultures expressed antigens of interest (**a**), narcolepsy sera were tested. Immunoreactivity was observed in the sera of 9 children (**b** and **c**). However, positivity did not correlate with orexin levels (**c** and **d**). 4/7 of the sera which bund to hippocampal neurons in preliminary experiments also had prior CBA/I.F. positivity (**d**).

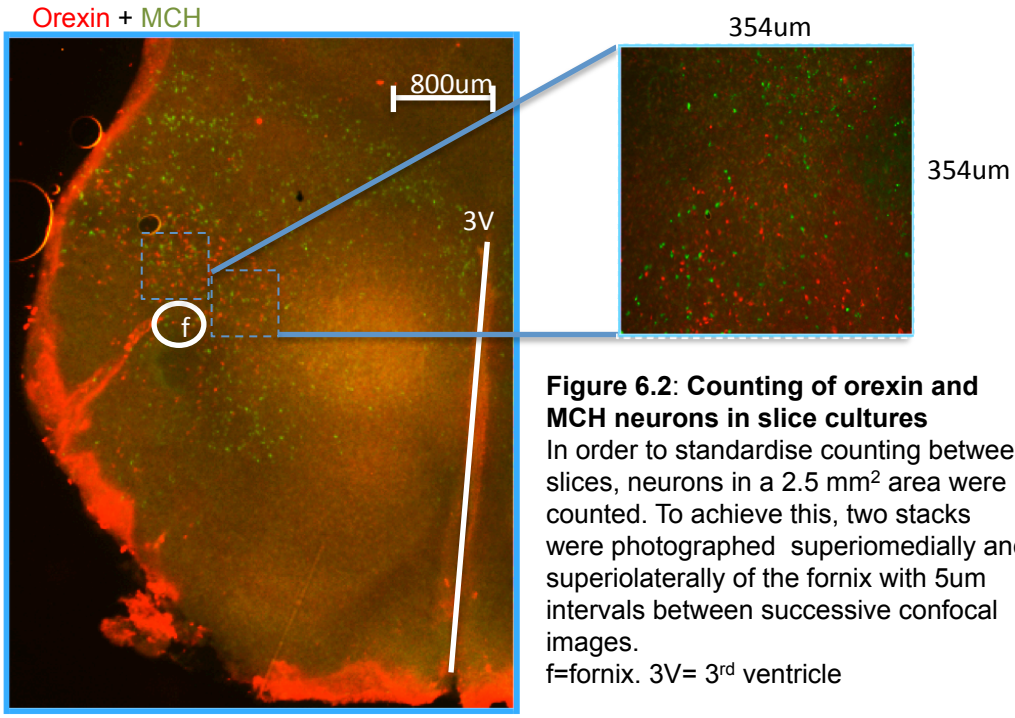


Figure 6.2: **Counting of orexin and MCH neurons in slice cultures**
In order to standardise counting between slices, neurons in a 2.5 mm^2 area were counted. To achieve this, two stacks were photographed superiomedially and superiolaterally of the fornix with 5um intervals between successive confocal images.
f=fornix. 3V= 3rd ventricle

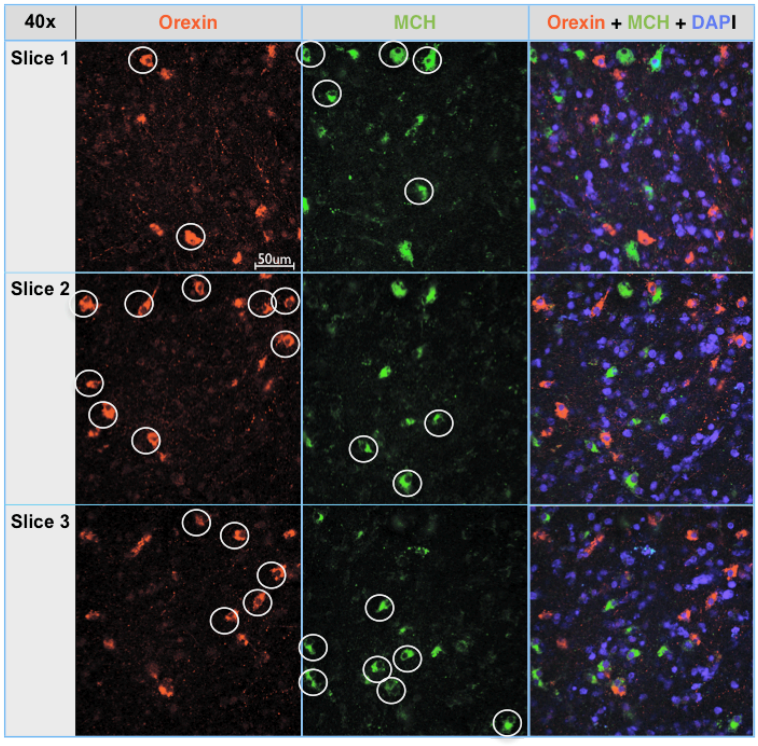


Figure 6.3: **Orexin and MCH neurons in successive 5um slices of rat hypothalamus organotypic cultures**
In order to prevent double counting of neurons appearing in more than one consecutive slice, neurons were only counted where the vacuolar DAPI-staining indentation of the nucleus was visible (circled in white).

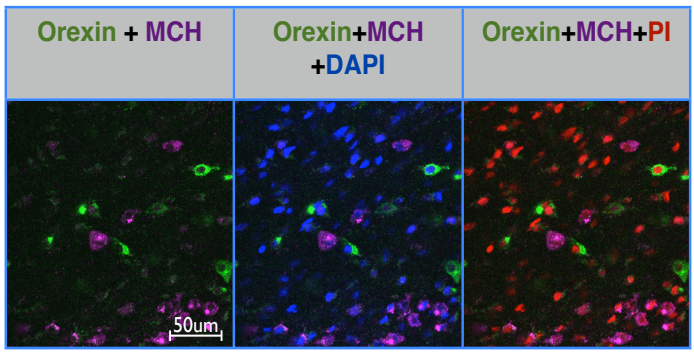


Figure 6.4: **Use of propidium iodide as a marker of neuronal viability:** PI was not found to be useful marker of neuronal viability in slice cultures a it became trapped at the base of sections, resulting in the staining of all nuclei.

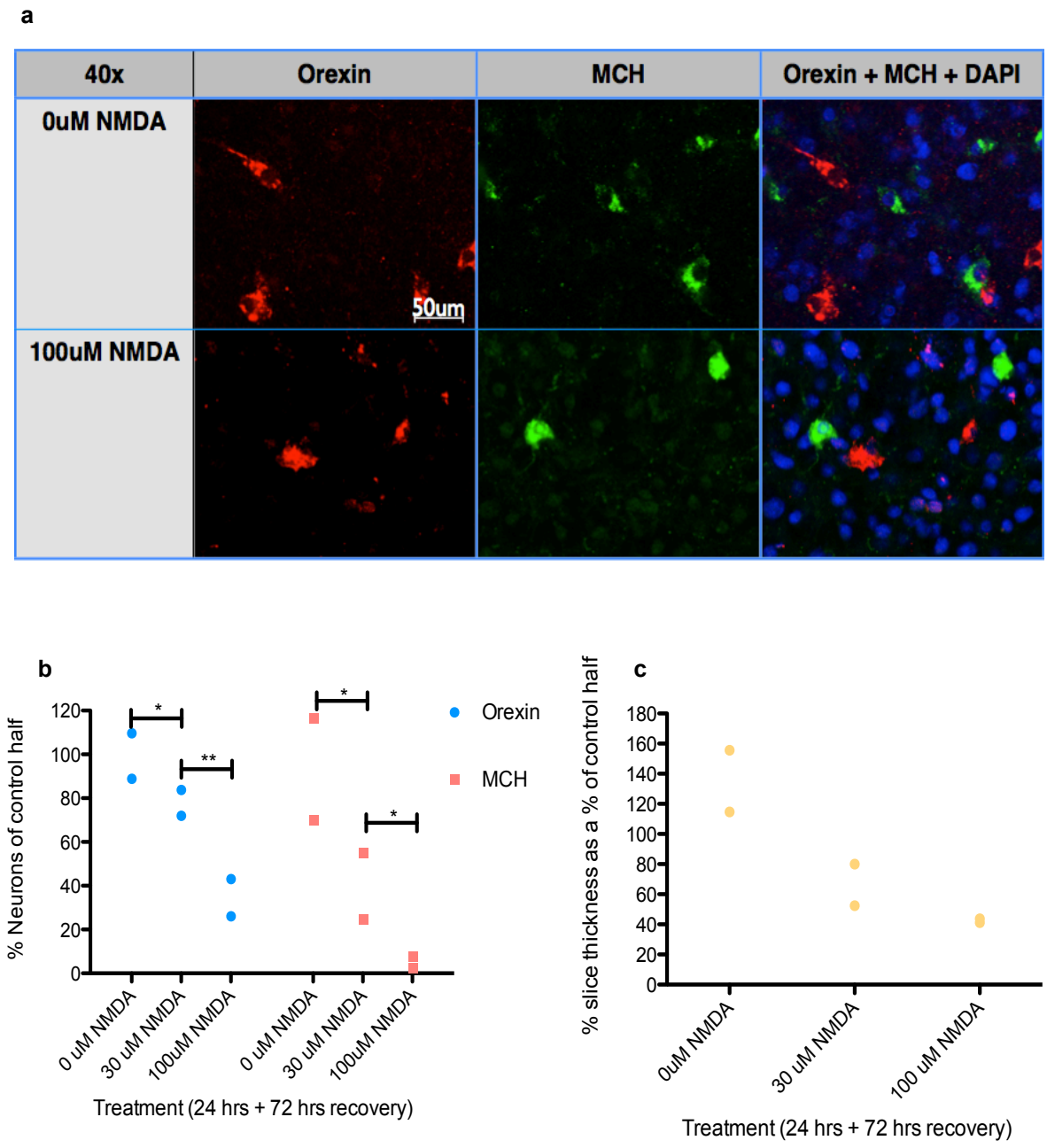


Figure 6.5: Effects of 24 hours incubation of slice cultures with NMDA followed by 72 hour post-incubation recovery. NMDA treatment appeared to result in neurons being smaller and more rounded with fewer processes (**a**). There were significantly fewer orexin and MCH neurons in test compared to control halves (n=2, **b**). These differences were reflected by significant differences in slice thickness compared to control halves (**c**).

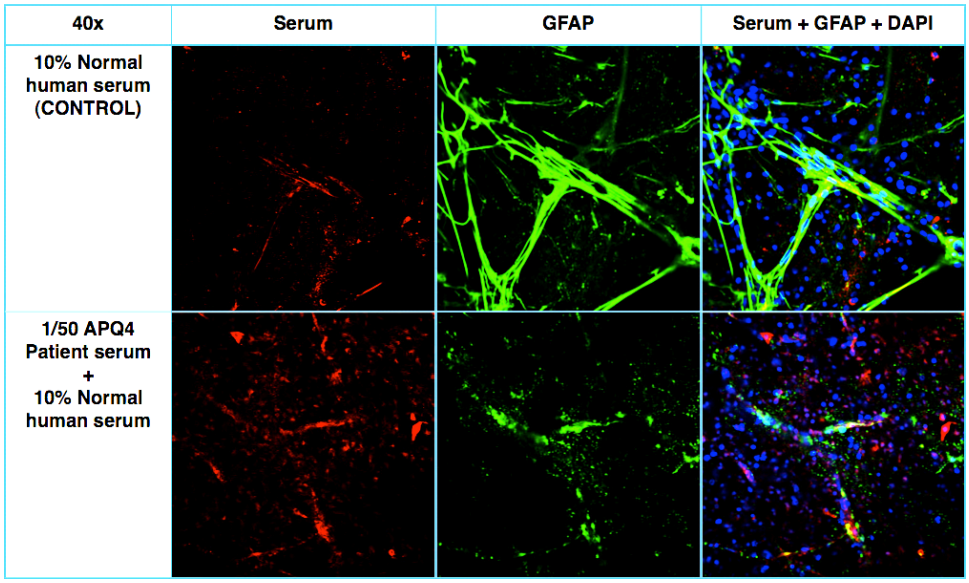


Figure 6.6: **Effects of overnight incubation of slice cultures with APQ4 patient serum (1/100) and human complement (serum from a healthy donor, 1/10)**. Patient binding to blood vessels and loss of GFAP astrocyte binding was observed with NMO patient serum, but not healthy control.

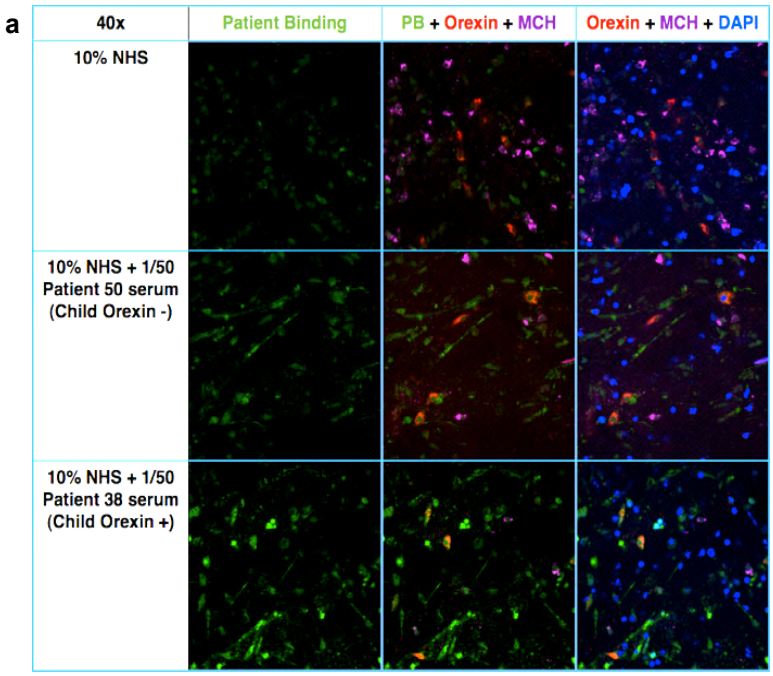


Figure 6.7: **Effects of 24 hours incubation of slice cultures with narcoleptic serum (1/100) and human complement (serum from a healthy donor, 1/10) followed by 72 hour post-incubation recovery**. Serum 51 ad 38 appeared to bind to both orexin and MCH neurons equally, whereas no binding was observed with healthy control serum (**a**). This was confirmed by calculation of mean fluorescence above background for orexin and MCH neurons in 4 images (**b,** mean ± SEM). Patient serum with complement didn't appear to kill orexin neurons, as demonstrated by comparing neuronal numbers in test and control halves (**c,**).

