Geneeskundige Stichting Koningin Elisabeth

Fondation Médicale Reine Elisabeth

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Geneeskundige Stichting Koningin Elisabeth

2006

Inleiding Verslag Activiteiten van de GSKE – FMRE

In het jaar 2006 zijn de gesubsidieerde onderzoeken verder gezet. De ploegen hebben hun rapporten overgemaakt en die zijn volledig te raadplegen op onze site.

Op 13 juni 2006 heeft Prinses Astrid, onze Erevoorzitter, verschillende prijzen uitgereikt zoals de UCB prijs (UCB award voor neurowetenschappelijk onderzoek) en de prijs Baron van Gysel de Meise voor neurowetenschappelijk onderzoek.
Deze prijzen ter waarde van respectievelijk 25,000 euro's en 12,500 euro's, werden in het Koninklijk Paleis uitgereikt door Prinses Astrid aan professor Pierre Vanderhaeghen van de U.L.B. en aan professor Rufin Vogels van de K.U.Leuven, in aanwezigheid van een groot aantal toehoorders bestaande uit vertegenwoordigers van de wetenschappelijke, politieke en academische wereld van ons land.

Deze gebeurtenis zal zich volgens de wensen van de mecenassen in de komende jaren herhalen. Het is een zichtbare vorm van aanmoediging en ondersteuning door de industrie en de privé mecenassen voor de neurowetenschappen.

In een ander domein heeft de stichting zijn steun verleend aan een onderzoek in samenwerking met het Ministerie van Volksgezondheid (KCE het federaal kenniscentrum voor gezondheidszorg). Het objectief was het inventariseren van de noden en de actuele beschikbare oplossingen voor jonge chronische neurologische patiënten (18-65 jaar) die lijden aan traumatische en vasculo-cerebrale aandoeningen, Multiple Sclerose en de ziekte van Huntington.

Dit institutioneel werk (UZ Gent, Ziekenhuis Inkendaal, V.U.B., G.S.K.E. en K.C.E.) wordt afgesloten met een belangrijk rapport met concrete voorstellen die zullen worden voorgelegd aan de bevoegde Minister. Het rapport zal begin 2007 overhandigd worden.

Op 19 december 2006, heeft Prinses Astrid een bezoek gebracht aan de laboratoria van professor Pierre Maquet van de ULg. Tijdens dit interessante bezoek, hebben de deelnemers kunnen kennis maken met de lopende onderzoeken in het domein van de slaap.

Na deze korte inleiding, dank ik de Voorzitter en de leden van de Raad van Bestuur die hun vertrouwen behouden in de onderzoeksploegen die hen door het wetenschappelijk comité worden voorgesteld. Hierbij dank ik ook alle leden van het wetenschappelijk comité.

Ten slotte wil ik in naam van de onderzoekers mijn erkentelijkheid betonen aan Prinses Astrid die met veel enthousiasme en zonder onderbreking de werkzaamheden van de Stichting volgt.

Prof. Dr. Th. de Barsy
Wetenschappelijk Directeur
Brussel, maart 2007

Fondation Médicale Reine Elisabeth

2006

Introduction Rapport d'Activités de la FMRE - GSKE

L'année 2006 a permis la poursuite des recherches subventionnées depuis le 1er janvier 2005. Les équipes ont présenté un rapport qui est entièrement disponible sur ce site.

Le 13 juin 2006, différents prix ont été remis par la Princesse Astrid, notre Présidente d'honneur. Il s'agit du prix UCB (UCB award pour la recherche en neurosciences) et le prix Baron van Gysel de Meise pour la recherche en neurosciences.
Ces prix ont une valeur respective de 25.000 euros et 12.500 euros et ont été remis au Palais Royal au Professeur Pierre Vanderhaeghen de l'U.L.B. et au Professeur Rufin Vogels de la K.U.Leuven par la Princesse Astrid, en présence d'une très large assemblée comprenant des représentants des autorités scientifiques, politiques et académiques de notre pays.

Cet événement, selon la volonté des mécènes, se reproduira au cours des années suivantes. Il constitue un encouragement visible et soutenu de l'industrie et des mécènes privés pour les neurosciences.

Dans un autre domaine, la fondation a soutenu un projet de recherches en collaboration avec le ministère de la Santé Publique (KCE, centre d'expertise fédérale). L'objectif était de faire l'inventaire des besoins et des solutions offertes actuellement aux patients neurologiques chroniques jeunes (18 à 65 ans) souffrants de séquelles de traumatisme crânien, d'accidents vasculaires, de multiple sclérose en "membres" et de maladie de Huntington.

Ce travail interinstitutionel (UZ Gent, Ziekenhuis Inkendaal, V.U.B.- F.M.R.E. et KCE) se terminera par un rapport important et des propositions concrètes seront présentées au Ministre compétent. La remise du rapport aura lieu début de cette année 2007.

Le 19 décembre 2006 la Princesse Astrid a visité le laboratoire du professeur Pierre Maquet à l'ULg. Cette visite intéressante a permis de familiariser les visiteurs avec les travaux en cours sur l'étude du sommeil.

Après ces quelques mots d'introduction je me permets de remercier le Président et les Membres du conseil d'administration qui maintiennent leur confiance dans les équipes de recherche qui leur sont présentées par le comité scientifique. Que ce dernier soit également remercié ici.

Au nom des chercheurs enfin, je tiens à exprimer toute ma reconnaissance à la Princesse Astrid qui, avec une écoute et un enthousiasme sans limite, accompagne sans relâche les travaux de la Fondation.

Prof. Dr. Th. de Barsy
Directeur Scientifique
Bruxelles, mars 2007

Universitaire ploegen gesteund door de Geneeskundige Stichting Koningin Elisabeth
Equipes universitaires subventionnées par la Fondation Médicale Reine Elisabeth
University Research groups supported by the Queen Elisabeth Medical Foundation

K.U.Leuven

Prof. Dr. P. Janssen
The functional interaction between dorsal and ventral visual stream areas during 3D object discrimination and grasping.

Prof. Dr. W. Vanduffel
Interactions between areas investigated using awake monkey fMRI.

Prof. Dr. R. Vogels
Coding of action categories in primate cortex.

U.A.

Prof. Dr. E. De Schutter
Experimental analysis of cerebellar coding.

Prof. Dr. V. Timmerman
Molecular genetics and biology of Charcot-Marie-Tooth neuropathies.

U.C.L.

Prof. Dr. A. Goffinet
Genetic, molecular and cellular mechanisms of cortical development.

Prof. Dr. J.N. Octave
Phosphorylation of the amyloid precursor protein intracellular domain: regulation of the production of ß-amyloid peptide and transcriptional activity?

Prof. Dr. E. Olivier
Distinct contribution of parietal and frontal cortical areas to the control of finger and hand movements.

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Prof. Dr. J. Tavernier
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Prof. Dr. P. Vanhoenacker
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Dr. S. Belachew
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Dr. L. Ris
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V.U.B.

Prof. Dr. Y. Michotte
In vitro and in vivo studies on the role of the IRAP enzyme/AT4 receptor system in learning and memory processes.

Progress Reports of the University Research Groups, supported by the Queen Elisabeth Medical Fondation in collaboration with the following Professors and Doctors (2006)

Progress Report of the Research Group of

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Results and Perspectives

Cdk2 loss preferentially affects adult SVZ progenitor cell development - comparison with early postnatal properties

The G1-S phase transition of the cell cycle is regulated by cyclin-dependent kinases and by their inhibitors. We have previously demonstrated that Cdk2 plays a crucial role in the control of oligodendrocyte progenitor cell (OPC) proliferation, in particular in the G1/S transition. We have also previously shown that, in adult (P90) neurogenic regions - the subventricular zone (SVZ) and ependymal layer of lateral ventricle - genetic deletion of Cdk2 affected cell proliferation, resulting in severe reduction in the number and the proliferation rate of NG2-expressing progenitor cells. Conversely, at early postnatal stages (P8-10), no differences between WT and $Cdk2^{-/-}$ mice were observed either in the total number of SVZ proliferating cells, or in th'ors ($Nkx2.2^{+}$) and neuroblasts (Dcx^{+}). *In vitro* studies using SVZ cell cultures demonstrated that neurosphere formation in adult cells obtained from $Cdk2^{-/-}$ mice was significantly decreased as compared to WT, whereas cell differentiation into oligodendrocytes, astrocytes and neurons was significantly increased. Conversely, no difference in neurosphere formation and cell differentiation was observed in cells from the early postnatal SVZ. Western Blot analysis in SVZ tissue demonstrated a significant up-regulation of Cdk4, Cdk6, Cyclin-D, Rb, E2F-1 and p21 protein expression in early postnatal $Cdk2^{-/-}$ tissue. On the other hand, levels of these proteins were comparable in the adult SVZ of WT and $Cdk2^{-/-}$ mice. A possible compensatory mechanism involving Cdk4 in SVZ cell proliferation is currently being studied by overexpression of Cdk4 in adult $Cdk2^{-/-}$ neurospheres. Our data indicate that Cdk4 might compensate the absence of Cdk2 in SVZ progenitors during early postnatal development, whereas this compensation does not occur in adult SVZ progenitor cells.

Adult hippocampal neurogenesis is reduced in Cdk2-deficient mice

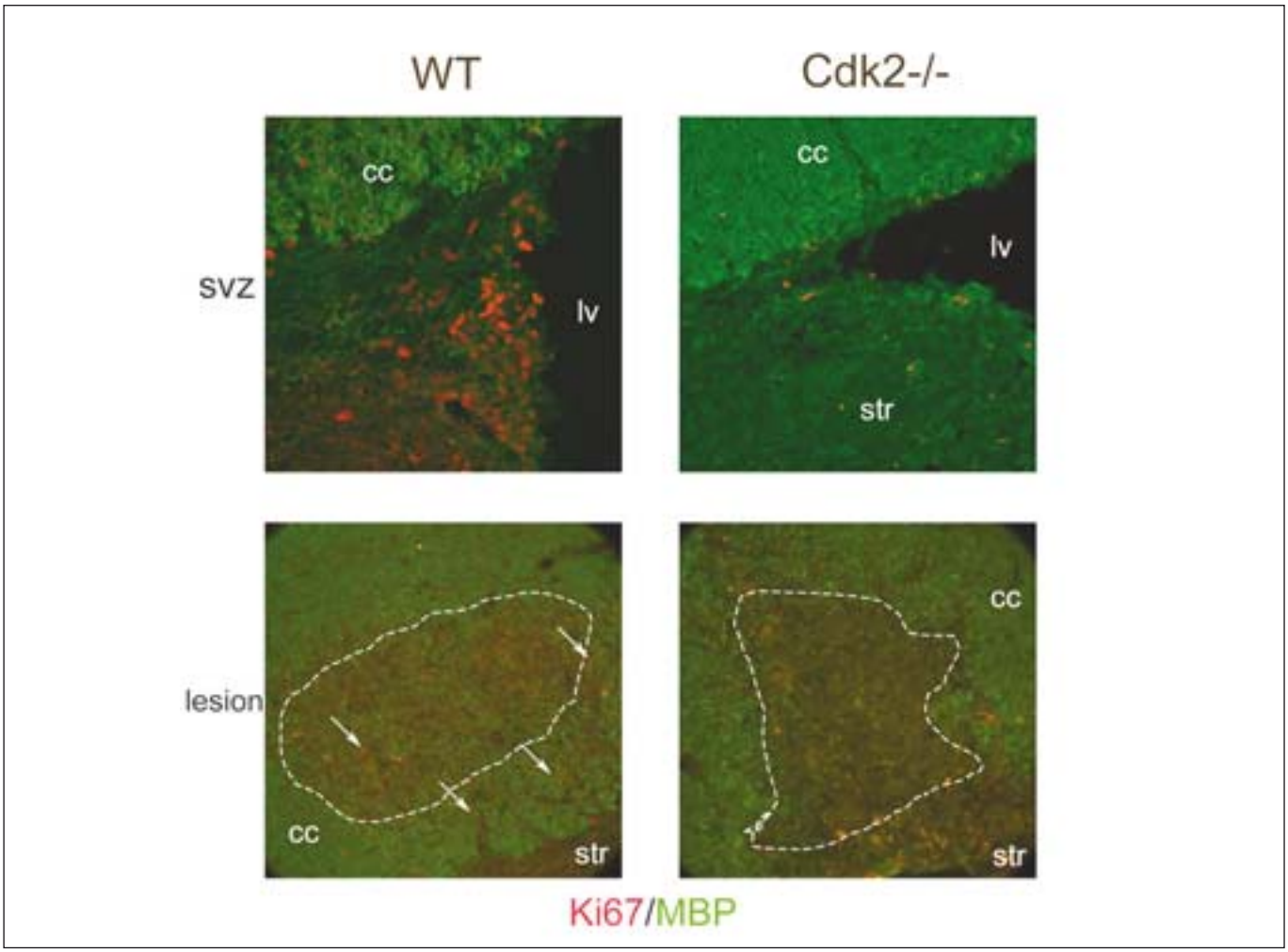
We studied the density of total ($Ki\text{-}67^{+}$) and S-phase dividing neural progenitors cells (NPCs) in the dentate gyrus of adult Cdk2-null mice, where neurogenesis persists throughout life. We observed that the lack of Cdk2 do not impede proliferation of NPCs in the adult dentate gyrus. However, the total amount of adult-born granule neurons in the dentate gyrus was drastically reduced in Cdk2-null mice. Altogether, our data showed that Cdk2 appears to control adult hippocampal neurogenesis without affecting NPC cell cycle progression. We are now currently addressing the hypothesis that Cdk2 loss might interfere with the survival of newly adult-born hippocampal neuroblasts by triggering cell cycle-unrelated apoptotic pathways.

Exploring the requirement of Cdk2 for normal white matter development and myelin repair following acquired demyelination

Type 2 cyclin-dependent kinase (Cdk2), which controls G1/S transition in eukaryotic cell cycle, was recently shown to be dispensable during embryonic development, since cdk2-null mice develop normally until adulthood. We assessed here the requirement of Cdk2 for proliferation of CNS precursor cells that generate newborn oligodendrocytes, but also neurons in specific

regions of the adult brain. We analyzed subcortical white matter, corpus callosum, striatum and cerebellar white matter areas with a broad spectrum of antigenic markers for distinct stages of oligodendroglial maturation (CNPase, MAG, Olig2). We found that the density and distribution of CNPAase-, MAG- and Olig2-expressing cells was identical in the corpus callosum and sub-cortical white matter of wild-type (WT) versus $Cdk2^{-/-}$ adult mice (3-months old). Similar results were obtained from the cerebellar white matter and striatum. Finally, the absolute density of Olig2-expressing cells (i.e. the entire oligodendroglial lineage cells) in the corpus callosum and of NG2-expressing OPCs in the cortex and in the corpus callosum was not different between WT and $Cdk2^{-/-}$ adult mice. Altogether, these data provide evidence that Cdk2 does not appear to be essential for normal developmental myelination. This indicates that the function of Cdk2 may be effectively compensated in neonatal OPCs (likely by Cdk4 overexpression as described above) whereas it could be crucial for cell cycle kinetics in adult OPCs.

We next used, as previously described (Nait-Oumesmar et al., 1999; Decker et al., 2002), a model of focal lysolecithin-induced lesion of the corpus callosum in order to challenge the role of Cdk2 in OPC proliferation and oligodendrogenesis following acquired non-autoimmune demyelination. Seven animals of each genotype (wt and $cdk2^{-/-}$ mice) deeply anesthetized with isofluran were positioned in a stereotaxic frame and injected unilaterally into the corpus callosum, using appropriate coordinates (1.5 mm anterior to the bregma, 1 mm lateral, and 2.2 mm deep from the skull surface) with a 5µl Hamilton syringe. Four animals of each genotype were injected with 2µl of a 1% lysolecithin solution (LPC, Sigma) in 0.9% NaCl with a flow rate of 0.5µl/min. Control animals (3) were injected with 2µl of saline solution only. The needle was kept in place for 2 min to reduce reflux up the needle track. The injection site was labeled with charcoal dust. To compare proliferation in response to demyelination between wt and $cdk2^{-/-}$ mice, 12 µm tissue sections were processed for Ki67 immunostaining and MBP (see figure below).



Evaluation of proliferation by Ki67 immunolabeling (red) A, B, in the SVZ, C, D in the lesion 7 days after LPC injection in the corpus callosum. The lesion is visualized by immunohistochemistry using anti-MBP (green). The arrows in C indicate the presence of few Ki67+ cells in the lesion of wild-type mice, none are present in the knock-out lesions.

Previous studies revealed that the percentage of $Ki67^{+}$ cells in the SVZ was lower in the $Cdk2^{-/-}$ mouse than in wt mouse. Our previous data also showed that demyelination enhances proliferation in the SVZ (Nait-Oumesmar et al., 1997, Picard-Riera et al., 2002). We therefore question whether this increase of cell proliferation in response to demyelination is influenced by the loss of Cdk2. This was indeed the case since in the SVZ of demyelinated animals, we observed a 2 fold increase of $Ki67^{+}$ cells in wt mice compared to $Cdk2^{-/-}$ mice. Demyelination is also known to trigger proliferation of glial cells in the lesion area. We found a 1.5 fold increase of $Ki67^{+}$ cells in wt mice compared to $Cdk2^{-/-}$ mutants. We are in the process of characterizing the proliferating cells with NG2 and CC1 immunostainings to identify oligodendrocyte progenitors, and GFAP to identify astrocytes. Our future experiments, will be to induce demyelination in a larger number of animals and to sacrifice them at different time points after LPC injection in order to monitor the dynamic of proliferation during the process of remyelination. In addition, we will evaluate and compare by immunostaining for MBP, the area of demyelination at different time points as a first assessment of the degree of remyelination between wild-type and knock out animals. These data will be confirmed in other series by electron microscopy.

Progress Report of the Research Group of

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Stochastic description of complex and simple spike firing in cerebellar Purkinje cells

Purkinje cells (PCs), the only output neurons of the cerebellar cortex, generate two distinct types of spikes, complex spikes (CSs) and simple spikes (SSs). In anesthetized rats CSs occur at very low frequencies (mean spontaneous firing rate: 1.3 Hz, range: 0.2 – 4.7 Hz), while SSs discharge at high firing rates (mean spontaneous firing rate: 27.9 Hz, range: 4.0 – 81.4 Hz) (Bower and Woolston, 1983; Vos et al., 1999; Brown and Bower, 2001). CSs are caused by activation of a climbing fiber, while SSs are assumed to be disynaptically triggered by mossy fibers via parallel fibers which are the axons of granule cells in the cerebellar cortex. It is likely that two anatomically separate afferent systems will transfer information in parallel. Although SS firing is known to be strongly influenced by CS, which usually cause a pause in SS firing (Ebner and Bloedel, 1981; Sato et al., 1992), CSs are generally assumed to occur independently from SSs.

Both CS and SS trains have been described as highly irregular, expressed by a high coefficient of variation (CV) (Vos et al., 1999)(Goossens et al 2001), but the underlying structure of the irregular spike trains is poorly understood. If consecutive interspike intervals (ISIs) are independent of each other, the theory of renewal processes can be used to investigate the underlying processes (Dayan and Abbott, 2001). Because of the independence of ISIs, spike trains generated by a renewal process are statistically fully specified by their ISI distribution function (Tuckwell, 1988). In other words, if spikes are generated by a renewal process, the ISI distribution will give the underlying process. For instance, if the ISI probability density function can be fitted by an exponentially decaying function, the process would be a Poisson process which is defined by a single parameter, the firing rate. If, more generally, a gamma distribution provides the best fit, the underlying process is called a gamma process, defined by two parameters, the so-called order and the mean firing rate.

In a previous study we showed that SSs recorded in anesthetized and awake rodents are not simple renewal processes, such as a Poisson process, because of the unexpectedly frequent occurrence of highly regular spike patterns (Shin et al., 2006). This, however, does not exclude that SS trains are composed of a mixture of multiple different renewal processes. Likewise, it is not at all clear yet, whether CSs can be described by a renewal process. In the present study we tried to fit spontaneous CS and SS activity recorded in anesthetized rats with mixtures of different renewal processes. Although many renewal processes have been studied (Tuckwell, 1988), we restricted ourselves here to one of the best known types, the gamma processes. Gamma processes are quite general, encompassing the Poisson process as a special case (order 1), and are relatively simple to handle. Moreover, the parameters of a gamma process can be easily linked to physiological parameters, regularity (order) and mean firing rate.

Characteristics of CS spiking patterns

We first established whether spontaneous CS trains observed in anesthetized rats can be considered as realizations of a renewal process. This requires that consecutive intervals, ISI_i and ISI_{i+1}, are statistically independent. If this is the case, a joint interval histogram of consecutive ISIs will be the same as that obtained by cross-multiplying two corresponding marginal histograms, because the marginal histogram (i.e. the conventional ISI histogram) does not contain any temporal order

information. To test this, we compared two joint histograms: one was the actual joint interval histogram counting the number of successive interval pairs in each bin (Figure 1A), and the other was obtained by cross-multiplying the ISI distribution, shown on the top (ISI_i) and right side (ISI_{i+1}) of Figure 1, with itself (Figure 1B). These two histograms were not significantly different ($p > 0.99$, χ^2 test), indicating that successive intervals were, indeed, independent and, hence, that CSs were generated by a renewal process.

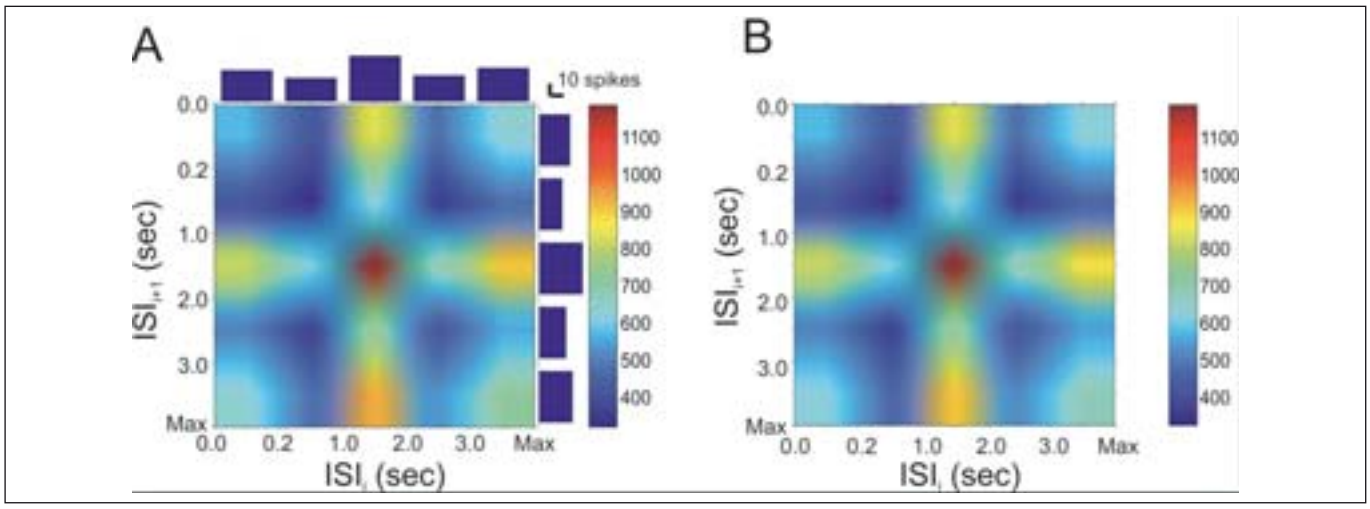

Figure 1: real (A) and shuffled joint interval histogram for complex spikes.

However, CS trains could not be described by a simple gamma process. In fact, most CS trains showed significantly higher mCV_2 values, 1.00 ± 0.02 (range: 0.71 ~ 1.30), than CV, 0.89 ± 0.02 (range: 0.76 ~ 1.22, $p < 10^{-6}$), implying that CS ISIs were more variable over short time ranges than for long-term averages. This is suggestive of a sudden switch of two very different distributions of ISIs. This was confirmed by inspection of the ISI distribution of CS trains, which clearly revealed a mixture of at least two ISI distributions, one with a narrow peak at around 100 ms, the other with a very broad peak between 1 and 2 sec. To separate these two distributions, we set a threshold at 400 ms, chosen by visual inspection. On average, 25.1 ± 1.7 % of ISIs were shorter than or equal to 400 ms. The corresponding frequency distributions were unexpectedly narrow; from 4 to 8 Hz (5.70 ± 0.13 Hz) for the group with short ISIs, and from 0 to 1 Hz (0.55 ± 0.03 Hz) for the other group. Note that no frequencies were observed around 2.5 Hz, the chosen threshold value. This confirms that there were indeed two clearly separated groups of ISIs in each CS train, one longer than or equal to 1 sec and the other shorter than 250 ms.

In the next step, we tested whether CS trains could be fitted by the random mixture of two gamma processes, separately obtained from groups of ISIs shorter or longer than 400 ms. First, both order and rate parameters were obtained for each group separately from the best fitting gamma distribution. Shorter ISIs exhibited higher orders (5.8 ± 0.4) than the longer ones (3.0 ± 0.1, $p < 10^{-6}$). The rate parameters were equal to the mean values of the ISIs divided by their estimated orders. From the two sets of estimated parameters from each CS train, i.e., two order parameters and the associated two rate parameters, two groups of random ISIs were generated and then, randomly mixed to obtain a final simulated CS train by permuting all random ISIs which were a

sequence of two groups of randomly generated ISIs. We found that these simulated CS trains were, indeed, very similar to the real ones (CC = 0.80 ± 0.03) and similar statistical properties like CV, mCV2, mean, and median of ISI.

A possible neuronal mechanism to generate the two groups of ISIs is depicted in Figure 2. Here, we assumed that the membrane potential of the inferior olivary neurons, the only source of climbing fiber activity, fluctuates with a mixture of two noisy frequency bands.

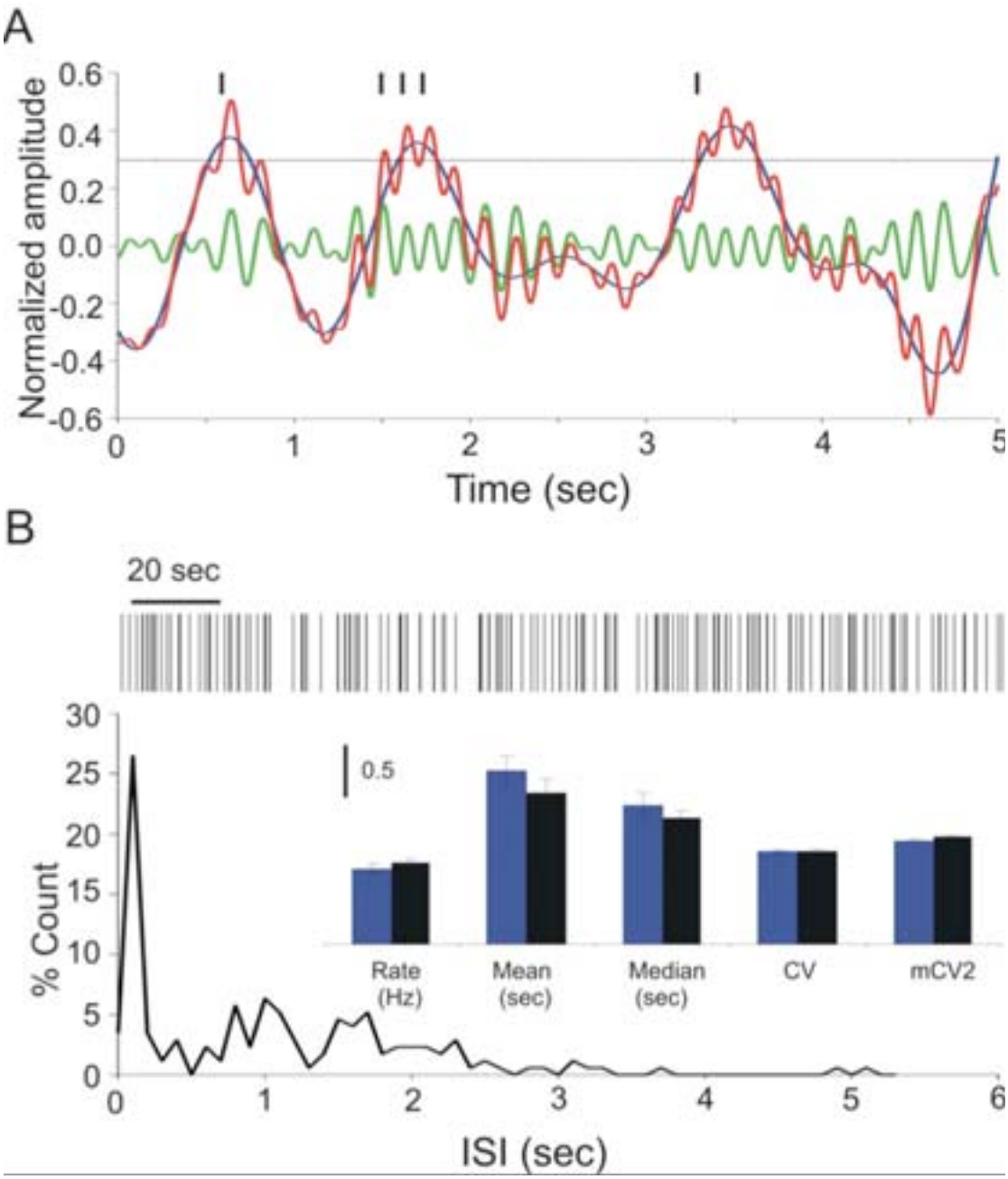

Figure 2: model can replicate the complex spike data.

These two frequency bands were generated by filtering white noise using two equiripple band-pass filters, ranging either from 4 to 8 Hz or from 0 to 1 Hz. The resulting combined oscillatory process depends on the relative gains (amplitudes) of the two component bands. By comparing the model with the data we found a unique set of parameters which for the entire set of CS recording data (N = 39) produced simulated CS spike trains in the physiological range. Figure 2A shows an example: the spikes generated from the summation (red) of two individual oscillations with gains of 0.2 (fast oscillation, green) and 0.8 (slow oscillation, blue) at the threshold of 0.3 (dashed line). The resulting simulated spike train (Figure 2B, inset) revealed very similar firing patterns and ISI histogram as the real data. The statistical properties (mean and median of ISI, CV, mCV_2) of the simulated spike trains were also similar ($p > 0.1$) (Figure 2B, inset), suggesting that the precise temporal structure of the two different gamma processes may not be important at least for spontaneous CS trains.

We showed that the short range variability found in CS trains can be explained by a mixture of two different oscillatory processes with very different frequencies; one with a frequency range from 0.5 - 1 Hz, the other ranging from 4 to 8 Hz. In fact, such division of frequency bands is already suggested by the ISI distribution of CS trains showing two clearly separated distributions. Moreover, we found that in order to get similar firing properties as those of CS trains recorded from anesthetized rats, the amplitude of the low frequency band should be some 5 times larger than that of higher frequency band. This suggests that in the anesthetized in vivo situation, an additional slow oscillatory process may modulate the faster oscillations observed in ION in vitro.

Our results are limited to the spontaneous activities recorded in anesthetized rats. In general, CS mean firing rates reported in awake animals are higher than those in this study (Keating and Thach, 1995; Lang et al., 1999). However, single trial traces of CS firing in awake monkeys show some shorter CS ISIs intermingled with longer ones (Keating and Thach, 1995). Thus, an interesting question is whether the higher mean firing rates of CS trains recorded in awake animals are caused by higher proportions of the high frequency band and whether different behavioral tasks increase specific frequencies.

Stochastic description of regular spike patterns and single intervals in SS trains

In a previous study, we reported that SS trains contain highly regular spike patterns, comprising series of 2 to 182 similar ISIs, which contain more than half of all SS ISIs (Shin et al., 2006). The ISIs not belonging to regular patterns were called singles and represented mostly the high-end tail of the ISI distribution. There, we also showed that SSs are not generated by a simple renewal process like a Poisson process. However, it remains possible that the ISIs in patterns are generated by a high order gamma process which renders it regular, while singles are generated by another, more irregular, i.e. lower order gamma process.

To test this hypothesis, we searched for possible gamma order parameters for regular patterns and singles separately. First, we computed the orders of regular patterns. Because we needed at least 20 ISIs to obtain the correct order estimate, we estimated orders only for patterns with more than 20 ISIs. This resulted in 1546 orders from 1.3 ± 0.6 % of all regular patterns. Surprisingly,

the distribution of orders (mean = 110.1 ± 1.7; median = 97.5), was itself a gamma distribution, with order parameter of 2.8 and rate parameter of 39.5. The orders of regular patterns were not related to their mean ISI. On average, more than 80 % of pattern mean ISIs were shorter than 20 ms. As short patterns (less than 20 ISIs) are otherwise statistically indistinguishable from long patterns (Shin et al., 2006), we assumed that short patterns are generated by a similar process as long patterns.

The interval distribution of single ISIs differed significantly from that of regular patterns ($p < 10^{-6}$, χ^2 test). Specifically, the singles comprised distinctly fewer short intervals. If all singles were generated by the same gamma process, the estimated order from the distribution of all singles should be equal to CV^{-2} (Gabbiani and Koch, 1998). We found, however, that the orders were significantly different from CV^{-2} ($p < 0.04$), indicating that singles are not caused by a single gamma process. This still leaves open the possibility that they were caused by a mixture of multiple processes. To estimate the orders of these presumed processes, we first took all singles from each SS train. For each of these we estimated the order for the first 20 singles, then moved to the next single and performed the next estimation, and repeated this procedure until the end of the singles which is a similar procedure to estimating rates using sliding windows. After each estimation, we checked if the obtained order estimate was 1 or larger and that it was similar to CV^{-2} within a 20% error range. In total, 29.3% (N = 5978) of the orders thus estimated satisfied this criterion. As the orders of the regular patterns, they also exhibited a gamma distribution (mean = 4.63 ± 0.03, median = 4.12), but with different parameters: order parameter 5.35 and rate parameter 0.87.

In contrast to CS firing which was shown to be a renewal process, SS firing cannot be a simple renewal process as it contains regular patterns which imply strong serial correlations (Shin et al., 2006). Thus, in the present study we focused on characterizing the stochastic properties of regular patterns and singles separately. The simplest scenario is that regular patterns are generated by gamma processes of higher orders, and singles by lower order processes, more similar to Poisson processes. As expected, regular patterns were about 20 times more regular than singles. Moreover, orders estimated from both regular patterns and singles were not fixed, but they showed a gamma distribution, suggesting that there are separate processes that independently regulate two distinct levels of regularity in SS firing.

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Progress Report of the Research Group of

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Genetic, molecular and cellular mechanisms of cortical development

Background

Work in our laboratory is focused on: i) The mechanism of action of Reelin and its signaling pathway; (ii) The development and use of *in vitro* systems to study neuronal migration to the cortex and the formation of the cortical plate; (iii) Role of the seven pass cadherin Celsr3 in brain development.

1. The Reelin signaling pathway.

The cloning of the Reelin gene, allowed the definition of a new signaling pathway that plays a key role in organizing the radial and laminar organization of the cortex (Tissir et al., 2002a; Tissir and Goffinet, 2003). Reelin is an extracellular matrix protein secreted by Cajal-Retzius cells (CR), that binds to two receptors of the lipoprotein receptor family named Very Low Density Lipoprotein Receptor (VLDLR) and Apolipoprotein-E Receptor Type 2 (ApoER2) expressed on the surface of migrating neurons. The cytoplasmic adapter Disabled-1 (Dab1) docks to the cytoplasmic tail of receptors and becomes tyrosine phosphorylated by Scr family kinases following Reelin binding to receptors (Jossin et al., 2003). Dab1 activation is further relayed in cortical neurons by various signaling pathways, particularly the PI3K, PKB/Akt (Bock et al., 2003) the Nck-beta and the CrkL-C3G-Rap1 pathways.

Reelin is processed *in vivo* into several products that result from partial cleavage at two sites located respectively between repeats 2 and 3 and between repeats 6 and 7. We showed that the central fragment (repeats 3-6) is necessary and sufficient to fulfill Reelin's function during brain development (Jossin et al., 2004). Two antibodies have been characterized extensively and allowed us to detect the predicted central fragment of reelin in tissues and in human cerebrospinal fluid. We have shown that these antibodies block the reelin signal. We showed that Reelin is cleaved by an enzyme secreted by embryonic neurons and have now evidence that cleavage is required for activity in vivo. This work is submitted for publication (Jossin, Gui, Goffinet, 2007).

In collaboration with the laboratory of J. Herz (Dallas), we showed that the PI3K and PKB/Akt kinases are implicated in Reelin signaling (Bock et al., 2003). An extensive study of this signaling pathway has been carried out and shows that PI3K mediates both reelin-dependent as well as reelin-independent developmental events. Akt/PKB is regulated by Reelin, like Gsk3beta and mTor, but none of them is involved in mediating the effects of reelin on cortical plate development. His pathway plays a key role in the Reelin-regulated growth of dendrites. These studies are at the final stage and will be submitted for publication in the coming months.

2. Study of neuronal migration to the cortical plate in vitro

Until recently, no *in vitro* system allowed the analysis of cortical plate (CP) development in vitro from the preplate stage. By systematic optimization of tissue culture parameters, we set up a system in which vibratome slices are prepared at the preplate stage (E13) and cultured for two days *in vitro*. In these conditions, CP development proceeds *in vitro* with features that

recapitulate its development *in vivo* (Jossin et al., 2003). We have used that system to screen a chemical library of 2000 molecules ("Diversity Set", provided by the National Cancer Institute, NCI), and identified eleven new molecules that interfere with migration in a manner that remains to be defined. The molecules are active in vitro but not in vivo, presumably because they do not cross the placental barrier and/or are degraded rapidly in vivo. Each of them corresponds to a potential target implicated in the regulation of neuronal migration and cortical development, and requires further study with development of chemical series of analogs. This work has been published (Zhou et al., 2006).

3. The role of the seven pass cadherin Celsr3 in cortical development

Celsr1-3 are a family of three mammalian gene orthologs of Flamingo (Tissir et al., 2002c; Tissir et al., 2002b). Celsr1 is expressed in zones of neural precursor proliferation and plays probably a role in the neuroepithelium, possibly by controlling planar polarity. Celsr2 is expressed in both the neuroepithelium and postmigratory neurons, and remains expressed in the adult brain. Celsr3 expression is restricted to postmigratory neurons, and is particularly high in the forebrain, including the CP. Mice with defective Celsr3 die shortly after birth of central ventilation failure. Their forebrain is highly abnormal, with no thalamocortical or corticostriatal connections, absence of internal capsule and of anterior commissure. They have no medial lemniscus, no corticospinal tract and profuse anomalies of longitudinal tracts in the hindbrain and spinal cord that account for the ventilation failure and neonatal death. The phenotype is identical to that generated by inactivation of frizzled-3 (Fzd3) ((Wang et al., 2002; Tissir et al., 2005; Wang et al., 2006a; Wang et al., 2006b).

We have made a conditional ("floxed") mutant mouse in which the region deleted in the Celsr3 -/- mice is flanked with loxP sites. Floxed Celsr3 mice are viable and fertile. We are using them in crosses with mice that express the Cre recombinase in forebrain (Foxg1-Cre), in cortical structures (Emx1-Cre) and study the anterior commissure and the internal capsule. We will shortly begin crosses with mice that express Cre in basal forebrain (Gsh2-Cre and Nkx2.1-Cre, collaboration with Dr Tekki-Kessaris, London). and in dorsal thalamus (Ror1-Cre, collaboration with D. O'Leary, Salk Institute).

We have initiated studies of the mechanism of action of Celsr3, based on a model inspired by the mechanism of PCP in Drosophila. Based on known function of Fzd3 and our in situ study of PCP-like gene expression in mice (Wang et al., 2002; Tissir and Goffinet, 2006; Wang et al., 2006a; Wang et al., 2006b), we think that Celsr3 may foster interactions between cells that express Fzd3 and Vangl2. We have raised antibodies against Celsr3 and cloned the cDNA coding for Celsr3, Fzd3 and Vangl2, with and without fluorescent tags.

4. Review on cortical development.

We have participated in reviewing two aspects of brain development, namely the role of Reelin and the issue of thalamocortical wiring (Forster et al., 2006; Price et al., 2006).

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The functional interaction between dorsal and ventral visual stream areas during 3D object discrimination and grasping.

We investigated the shape selectivity of individual neurons in the posterior parietal area LIP of awake behaving rhesus monkeys. Three single-cell studies were started that will provide valuable information for the investigation of the functional interactions between inferior temporal and posterior parietal cortex. A fourth study investigates the interaction between area LIP and the frontal eye fields (FEF), using a paradigm with chronically implanted electrodes in the FEF for microstimulation and simultaneous single-electrode recordings in area LIP during a delayed saccade task.

In a first study, LIP neurons were tested with simple, two-dimensional (2D) shapes during passive fixation. The shapes varied along one dimension, known to be important for grasping (curvature, aspect ratio, orientation, etc.). Most neurons in the anterior part of LIP (aLIP) were tuned for at least one of the 2D shape dimensions: 101 out of 117 neurons tested (86%) showed significant response differences during the presentation of the 2D shapes. The latency of the neuronal responses was surprisingly short: response onset occurred as early as 30-40 ms after stimulus onset, and differential responses between preferred and nonpreferred shapes emerged 40-50 ms after stimulus onset. To determine to what extent LIP neurons are position invariant for 2D shape we tested 50 LIP neurons tuned for 2D shape with their preferred shape and a nonpreferred shape at 35 positions spaced 2 degrees apart in the contralateral hemifield. Only a limited proportion of the neurons (32%) preserved their selectivity at different positions. The majority of the neurons (52%) showed responses to the nonpreferred shape at a different position that were equal to or even larger than the response to the preferred shape at the position in the original test, which indicates that the apparent 2D shape selectivity arises from interactions between the position of the stimulus and the shape of the receptive field of the neuron. The remaining neurons (12%) showed complex interactions between shape and position. Hence LIP neurons signal both the 2D shape and the position of stimuli presented during passive fixation. The shape representation in LIP, however, seems to be radically different from the shape representation in the inferior temporal cortex.

A second study investigated the selectivity of posterior parietal neurons for three-dimensional (3D) shapes, in which depth is defined by gradients of binocular disparity. Stimuli and metrics were exactly the same as in our previous studies in the inferior temporal cortex (IT) (Janssen et al., 2000). We recorded 91 single neurons in AIP, an area in the lateral bank of the Intraparietal Sulcus known to be important for grasping, in three passively fixating rhesus monkeys. A large proportion of the neurons (48, 53%) displayed selectivity for the 3D shape of the stimuli that could not be accounted for by the pattern of the monocular responses. The latency of the 3D shape selectivity was 90 ms, which is shorter than that observed in IT (120 ms). Most neurons (36/48, 75%) preserved their selectivity over a number of positions-in-depth, indicating that these neurons are responding to the spatial variation of disparity and not just to differences in the position-in-depth of the stimulus. We determined the sensitivity of posterior parietal neurons by testing 3D shape selective neurons with concave and convex surfaces of varying disparity amplitudes (from 1.3 to 0.03 degrees). The tuning for 3D shape was monotonic in the large majority of the neurons, and

we did not observe a sharp drop in the response between the two smallest amplitudes (-.0.3 and +0.03), as was the case in IT. Finally, most neurons showed invariance of the 3D shape selectivity for a limited number of positions in the frontoparallel plane and for different sizes (2.5 to 8.5 degrees). Hence the representation of 3D shape in the posterior parietal cortex may be different from the representation in the inferior temporal cortex. The data collection for the two previous studies has been almost completed. Manuscripts are expected to be submitted in the first half of 2007.

In a third study, we prepared two rhesus monkeys for recording and inactivation studies to determine to what extent posterior parietal and inferotemporal neurons interact during the discrimination of 3D shapes. These animals were trained on a 3D-shape discrimination task in which the monkeys have to make an eye movement to the right when the stimulus is a convex surface and to the left when the stimulus is concave. Task difficulty is manipulated by changing the percentage of the dots that give rise to the percept of the curved surface (between 0% and 100% coherence). We determined whether the trial-by-trial variation in the neuronal firing correlated with the monkeys' reported percept of the 3D shape of the stimulus, which can captured by a metric termed the choice probability. Recordings in one monkey have started recently, and initial results suggest that most AIP neurons show positive choice probabilities that are significantly larger than chance. These results indicate that the activity of AIP neurons correlates positively with the reported percept of the animals. In the second half of 2007, recordings will start in the inferior temporal cortex of the same animals, so that the choice probabilities in AIP and IT can be compared directly. Inactivations during these recordings will allow to assess the contribution of each area to 3D shape discrimination.

Finally, we started to investigate to what extent the attentional modulation in the activity of LIP neurons originates in the Frontal Eye Fields (FEF). We trained two rhesus monkeys in a delayed saccade task, in which the animals have to make an eye movement to a blue target dot as soon as a second spot (the go-cue) changes luminance (either brightening or dimming). Next we implanted two rhesus monkeys with 40 electrodes in the FEF and prepared the animals for single-cell recordings in area LIP. In at least 30 of the implanted electrodes, microstimulation reliably evoked saccadic eye movements. During the delayed saccade task, either the target or the go-cue can be presented in the receptive field of the LIP neuron, and microstimulation (currents less than 50% of the threshold for evoking saccades) is applied in 50% of the trials on an FEF electrode in which the response field is at the same location as the LIP receptive field. The artifact that originates from the microstimulation currents is subtracted from the recording signal by means of an artifact zapper (Riverbend Instruments, Birmingham AL). Initial results indicate that FEF microstimulation increases the responses of LIP neurons when the go-cue is presented in the receptive field of the LIP neuron, but not when the target is in the receptive field. Hence FEF neurons increase the visual response but not the motor response of LIP neurons.

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Human Brain Function in Sleep.
Studies in Man by Multimodal Functional Neuroimaging

1. Introduction

Our project aims (i) at further characterizing the neural correlates of human sleep and (ii) at specifying the functional relevance of sleep processes for waking brain function and cognition, at the systems levels, using combined EEG/fMRI acquisitions.

2. Neural correlates of human sleep

Neural correlates of spindles

The neural correlates of slow (11-13 Hz) and fast (13-15 Hz) sleep spindles were characterized using simultaneous electroencephalography and functional magnetic resonance imaging (EEG/fMRI) recordings in non sleep-deprived healthy human participants. The thalami, paralimbic areas (anterior cingulate and insular cortices) and auditory cortices increased their activity in relation to both spindle types. In thalami, the activity specifically related to slow and fast spindles was partially segregated and preferentially involved medial dorsal and ventral posterior lateral thalamic regions, respectively. However, no difference was detected in the direct comparison between slow and fast spindles. In contrast, there were significant differences in cortical responses to slow and fast spindles. The activity related to slow spindles was mainly limited to the common spindle response pattern, although significantly larger in superior frontal gyrus as compared to fast spindles. Fast spindles recruited cortical regions involved in sensori-motor processing, as well as hippocampal and mesiofrontal regions. These data suggest that slow and fast spindles are not primarily related to the presence of two distinct thalamic populations oscillating at different frequencies but to the modulation of thalamic populations by partially segregated cortical networks with different oscillatory properties.

Neural correlates of slow waves

The two main stages of human sleep, i.e. Rapid-eye-movement (REM) and Non-rapid-eye-movement (NREM) sleep, display drastically different patterns of brain activity : while REM sleep has been characterized as an active state in terms of metabolic changes, NREM sleep has been shown as a hypoactive state during which the brain undergoes a regional decrease of metabolism[1]. This view of deepest sleep stages as a quiescent state is challenged by the oscillatory nature of NREM sleep, during which the predominant activity consists in slow oscillations (SO). The latter are characterized on a cellular level by a slow alternation of cortical hyperpolarization and depolarization phases[2]. Here we used simultaneous electroencephalography (EEG) and functional magnetic resonance imaging (fMRI) recordings during NREM sleep in non-sleep deprived normal human subjects, to show that the brain activity is enhanced during NREM sleep. The increased activity is time-locked to depolarization phases of SO and is distributed in specific brain areas including thalamus, medial prefrontal and auditory cortices, hippocampus, ponto-mesencephalic tegmentum and cerebellum. These results constitute the systematic report of active brain processes during NREM sleep on a macroscopic level in humans, and suggest that SO are locally-generated cortical processes

modulated by distant cortical and subcortical structures. Moreover, they may also reflect functional inter-relationships underlying brain plasticity and memory processes during NREM sleep. This profound reappraisal of the functional neuroanatomy of NREM sleep gives significant insight into sleep mechanisms and functions, and emphasizes that "quiescent" sleep stages are not states of pure brain restful recovery but periods of intense neuronal activities shaped by the SO.

3. Influence of post-training wakefulness, sleep and lack of sleep on learning and memory

Our aim is (i) to characterize the processing of the memory trace during the post-training waking period and (ii) to characterize the effects of sleep deprivation on the sleep-related memory processing

The typical experimental design in this subproject will be to expose the participants to an (explicit or implicit) memory task and retest them on the same task after various delays during the immediate post-training waking period, after one night of sleep or after sleep deprivation. In some cases, both total and partial sleep deprivation will be considered.

Effect of sleep and lack of sleep in spatial memory

Sleep deprivation is believed to hinder a process of consolidation during which recently acquired, hippocampus-dependent, spatial memories are integrated into permanent cerebral networks. Using functional magnetic resonance imaging, we mapped regional cerebral activity during place finding in a virtual town, immediately after learning and three days later, in subjects either allowed to sleep or sleep-deprived on the first posttraining night. In all participants, delayed place retrieval was characterized by decreased activity in the hippocampus and increased activity in the caudate nucleus. Sleep on the posttraining night further enhanced caudate nucleus activity at delayed retrieval, and modulated its relationship with behavioral performance. Equipotent levels of performance at delayed retrieval were attained both after sleep and sleep deprivation, suggesting that sleep on the first post-training night favours a shift of brain activity on the long term so that initial effortful navigation, mostly based on a hippocampus-dependent spatial strategy, later on engages a more automatic cognitive strategy mediated by striatal regions.

These data are now published in PNAS.

Effect of sleep and lack of sleep in emotional memory

Using fMRI, we characterized the neural correlates of successful recollection of emotional and neutral stimuli 72 hours after encoding, with or without total sleep deprivation during the first post-encoding night. In contrast to recollection of neutral stimuli which was deteriorated by sleep deprivation, similar recollection levels were achieved for negative stimuli by recruiting different non overlapping cerebral networks, depending on whether the subjects were allowed to sleep on the first post-encoding night or not. In the sleep deprived group, recollection of negative items elicited responses in the amygdala and an extrastriate area, a pattern suggestive of an incompletely consolidated memory. In contrast, in the sleep group, recollection of negative stimuli recruited

a large set of cortical areas, including the medial prefrontal cortex and the hippocampus. This finding suggests that the emotional significance of the encoded information influences the offline memory processing during sleep on the first post-encoding night, and enhances the interplay between hippocampal structures and neocortical areas.

These results are submitted for publication.

Offline processing of motor memory

Motor skill learning is a dynamic process that continues covertly after training has ended and eventually leads to delayed increments in performance[1]. With the finger-tapping task, it is thought that little improvement can occur during post-training wakefulness, a significant gain in performance being observed only after sleep[2-5]. Here, we show that performance temporarily improves early on, 5 to 30 minutes after training. Although this early boost in performance is no longer present 4 hours later, it is predictive of performance levels eventually achieved 48 hours later in the absence of any further practice. Low-frequency repetitive transcranial magnetic stimulation (rTMS) applied over the primary motor cortex (M1) immediately after learning depresses this early boost in performance but does not affect the delayed improvement observed 48 hours later. These results recast our understanding on consolidation of motor memories in humans, sorting out an early but short-lived boost in performance from the genuine enduring consolidation process.

These results are now published in Learning and Memory.

Offline processing of oculomotor memory

A motor sequence is usually learned through repeated practice. However, even after a single training session, motor performance continues to improve in the absence of any further practice, suggesting that memories are processed offline. Offline memory processing underpins memory consolidation, which progressively transforms the initial fragile trace into a robust memory available in the long term.

Functional magnetic resonance imaging was used to explore the cerebral correlates of implicit oculomotor sequence learning, both during training and during a single test session taking place 30 minutes, 5 or 24 hours later. Performance during training distinguished slow from fast learners and interestingly, only fast learners improved overnight. During training, responses in the hippocampus differed between slow and fast learners. In the latter, the hippocampal responses were linearly related to the overnight gain in performance, suggesting that an early hippocampal recruitment during training conditions subsequent motor memory consolidation. During the first 24 post-training hours, memory consolidation in fast learners was characterized by the emergence of cerebellar, striatal and hippocampal responses in a distinct and ordered sequence. Importantly, the interactions between hippocampus and striatum changed over time. The negative functional connectivity detected during training was not longer observed, and even turned to cooperation overnight when memory trace is consolidated.

These results are submitted for publication.

Offline processing of spatial and procedural memories during the immediate post-training wakefulness

Much remains to be discovered about the fate of recent memories in the human brain. Several studies have reported the reactivation of learning-related cerebral activity during posttraining sleep, suggesting that sleep plays a role in the offline processing and consolidation of memory. However, little is known about how new information is maintained and processed during posttraining wakefulness before sleep, while the brain is actively engaged in other cognitive activities.

We show using fMRI that brain activity elicited during a new learning episode modulates brain responses to an unrelated cognitive task during the awake period that follows the end of practice. This posttraining activity evolves in learning-related cerebral structures, in which functional connections with other brain regions are gradually established or reinforced, and correlates with behavioral performance, following a different time course for hippocampus-dependent and hippocampus-independent memories.

Our experimental approach allowed to characterize the offline evolution of the cerebral correlates of recent memories, without the confounding effect of concurrent practice of the learned material. Results indicate that the human brain extensively processes recent memories already during the first hours of posttraining wakefulness, even when currently coping with unrelated cognitive demands.

These results are published in PloS Biology.

4. Non Image Forming (NIF) brain responses to light

Study 1 : white light

Light enhances both alertness and performance in humans. These effects do not correspond to classical visual responses to light but represent Non-Image Forming (NIF) responses, which involve a recently described system of photoreception. Light also profoundly influences regional brain function, beyond classical visual responses. Many studies focused on night time light exposure, but little is known about the daytime responses, especially their neural correlates and temporal dynamics.

Using fMRI, we characterized the neural correlates of the alerting effect of daytime light by assessing the responses to an auditory oddball task before and after exposure to a bright polychromatic white light. Light-induced improvement in subjective alertness was linearly related to an increased responsiveness in the posterior thalamus. In addition, light enhanced responses in a set of cortical areas involved in the oddball paradigm, preventing decreases of activity otherwise observed during continuous darkness. Importantly, the increases in response declined within minutes after the end of the light stimulus, following various regionally-specific dynamics. These findings suggest that light can modulate the activity of subcortical structures involved in alertness, thereby dynamically promoting cortical activity in networks involved in ongoing non-visual cognitive processes.

These results are published in Current Biology

Study 2 : monochromatic lights (blue vs green)

Besides classical visual effects, light elicits non-visual brain responses which profoundly influence physiology and behavior. These effects are mediated in part by a recently discovered, blue light sensitive photoreceptor system. At present, there is no evidence that blue light exposure is effective in modulating non-visual brain activity related to complex cognitive tasks, especially when administered during daytime. Using functional magnetic resonance imaging (fMRI), we show that, while participants perform an auditory working memory task, a short (18 minutes) daytime exposure to a blue (470nm) or green (550nm) monochromatic light ($3x10^{13}$ photons/cm^2/s) differentially modulates regional brain responses. The blue light typically enhanced brain responses or at least prevented the decline otherwise observed with the green light in frontal and parietal cortices implicated in working memory, and in the thalamus involved in the modulation of cognition by arousal. Our results imply that a low amount of monochromatic light can affect cognitive functions almost instantaneously depending on the spectral quality of the light exposure, even during daytime.

These results are submitted for publication.

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In vitro and *in vivo* studies on the role of the IRAP enzyme/AT_4 receptor system in learning and memory processes

I. Introduction

1. Ang IV and the IRAP enzyme /AT4 receptor system

The renin-angiotensin system (RAS) is widely recognised as the most powerful signalling system for controlling sodium balance, body fluid volumes and arterial blood pressure. The major RAS peptide is the octapeptide angiotensin II (Ang II) and most of its effects are triggered by AT_1 receptor activation. However, shorter peptide fragments such as Ang-(1-7), Ang III and Ang IV were shown to be bioactive as well. In this respect, the hexapeptide Ang IV sparked great interest because of its facilitatory role in memory acquisition and retrieval.

Ang IV binds only with low affinity to AT_1 receptors. Yet, as most of its physiological effects are already observed at nanomolar concentrations, it is generally accepted that these are mediated via a specific "AT_4 receptor" subtype (de Gasparo et al., 1995, 2000). The pharmacological profile of the AT_4 receptor deviates significantly from that of AT_1 and AT_2 receptors. These putative "AT_4 receptors" are activated by Ang IV and synthetic peptide analogues such as Norleucine^{l}-Ang IV (Nle^{l}-Ang IV) and Norleucinal (Chai et al., 2000, Albiston et al., 2001). They also constitute cellular targets for hemorphins, such as LVV-hemorphin-7 (LVV-H7), endogenous CNS peptides obtained by hydrolysis of the beta chain of hemoglobin (Møeller et al., 1997).

Intriguingly, in 2001, the "AT_4 receptors" were identified as insulin-regulated aminopeptidase (IRAP) or cystinyl aminopeptidase (EC 3.4.11.3), also known as placental leucine aminopeptidase (P-LAP) and oxytocinase (Otase) (Albiston et al., 2001). IRAP is a type II integral membrane protein homologous to aminopeptidase N (AP-N), and other Zn^{2+}-dependent enzymes of the gluzincin aminopeptidase family (Rogi et al., 1996, for review see Vauquelin et al., 2002). There is also evidence that in addition to IRAP, AP-N may also mediate some of the effects of Ang IV (Garreau et al., 1998).

2. Role of Ang IV in memory and learning

Initial interest in Ang IV originated from its ability to increase memory acquisition and recall in passive and conditioned avoidance response studies (Braszko et al., 1998, Wright et al., 1993, 1996, Tchekalarova et al., 2001). Intracerebroventricular (i.c.v.) administration of the AT_4 agonist Nle^{l}-Ang IV facilitated the ability to solve a spatial learning task in the circular water maze, an effect that was blocked by the putative "AT_4 antagonist" Divalinal-Ang IV (Wright et al., 1999). Divalinal-Ang IV also counteracted scopolamine-induced disruption of spatial learning (Pedersen et al., 1998). Moreover, Ang IV and LVV-H7 facilitated potassium-evoked acetylcholine release from rat hippocampal slices (Lee et al., 2001), suggesting an Ang IV-acetylcholine interaction.

Electrophysiological and biochemical studies revealed that the cognitive effects of AT_4 agonists are mediated via the hippocampus. Ang IV and its analogues significantly enhanced hippocampal long-term potentiation (LTP) in the dentate gyrus and the CA1 field, both *in vitro* (Kramar et al., 2001) and *in vivo* (Wayner et al., 2001). Moreover, autoradiographic studies revealed that AT_4 binding

sites are prominent in brain structures important to cognitive processing, including hippocampus (Miller-Wing et al., 1993).

3. Working hypotheses and specific aims of the project

The above findings imply that Ang IV might be a ligand for the putative AT_4 receptors as well as a competitive inhibitor of IRAP's catalytic activity (Albiston et al., 2004). We are performing a critical evaluation of the working hypotheses that IRAP is indeed the AT_4 receptor, and that the IRAP enzyme/AT_4 receptor system represents a major cellular recognition and signalling site for Ang IV in the CNS. This research is of special interest in the field of cognition and it may contribute to our understanding of pathophysiological conditions such as Alzheimer's disease.

Homodimer formation is one of the characteristic features of the membrane-bound M1 metallopeptidase family (Shipp and Look, 1993) to which IRAP belongs. As dimers, these enzymes have the potential to convey information across cell membranes in the same way as growth factors and cytokine receptors. In this respect, the structurally related AP-N (EC 3.4.11.2) and dipeptidyl-peptidase IV ectoenzymes have already been shown to mediate intracellular signalling (Gaetaniello et al., 1998, Navarette Santos et al., 2000). Therefore we will investigate the capability of the IRAP enzyme/AT_4 receptor system, after binding with Ang IV, to trigger intracellular signalling pathways in neuronal cells.

Despite the fact that Ang IV appears to play a role in memory acquisition and learning, there are no *in vivo* data demonstrating the modulation of neurotransmitter release by Ang IV. Therefore, *in vivo* microdialysis will be used to unravel possible effects of Ang IV on the extracellular levels of acetylcholine, glutamate and other neurotransmitters in brain regions involved in learning and memory processes. These experiments will be performed in rats, wild-type mice and IRAP knock-out mice to give a decisive answer whether the effects of Ang IV are elicited only by interaction with the IRAP/AT4 system.

Since the major *in vitro* substrates of the IRAP enzyme, oxytocin, vasopressin and somatostatin, are known to play an important role in cognitive function (Kovacs et al., 1994, Matsuoka et al., 1995, Alescio-Lautier et al., 2000), it was proposed that Ang IV and other AT_4 receptor ligands could mediate at least part of their physiological effects by inhibiting IRAP's enzymatic activity (Albiston et al., 2001). In this project, we will investigate if inhibition of IRAP by Ang IV will indeed result *in vivo* in an increased extracellular concentration of these neuropeptides of interest. Moreover, selective oxytocin, vasopressin and somatostatin receptor antagonists will be used to block physiological effects induced by Ang IV or its analogues.

These new concepts offer a wide range of original opportunities for examining the physiological roles of the "IRAP/AT_4" system as well as the mechanisms of action of Ang IV within learning and memory processes.

II. Report 2006

1. Exploring the presence and function of the IRAP enzyme/AT4 receptor system and AP-N in vitro

In collaboration with Prof. T. Hallberg (University Uppsala, Sweden) and with Prof. D. Tourwe (Department of Chemistry, Vrije Universiteit Brussel), we obtained a **series of novel Ang IV analogues** for *in vitro* screening with respect to their stability, potency and selectivity for human IRAP transiently expressed in HEK293 cells. In this respect, the AT_4 receptor binding activity of a large series of linear Ang IV analogues were previously disclosed (Handa et al., 1999, Krishnan et al., 1999, Lee et al., 2003). However, we amongst others now clearly established that these experiments only provide information about the ability of these peptides to interact with the inactive IRAP apoenzyme (i.e. enzyme lacking the catalytic Zn^{2+}) (Lew et al., 2003; Demaegdt et al., 2004, Laeremans et al., 2005). To obtain physiologically more relevant information about the new Ang IV analogues, we first focussed on enzyme-based assays. Accordingly, their potency and IRAP versus AP-N selectivity was estimated by monitoring their ability to dose-dependently inhibit the respective aminopeptidase catalytic activities (based on the cleavage of the substrate L-leucine-p-nitroanilide). IRAP versus AT_1 receptor selectivity was then estimated by assessing the ability of the peptide analogues to compete with [^{3}H]valsartan to the human AT_1 receptors stably expressed in CHO cells. Finally, stability of the peptide analogues towards breakdown by metalloproteases present in CHO-K1 cell membranes was estimated by comparing their competition binding curves either upon co-incubation with the radioligand [^{125}I]-Ang IV (no breakdown since the assay was performed in the presense of peptidase inhibitors) or after preincubation with the membranes in medium only. The already obtained data are highly encouraging since both chemistry teams managed to produce peptide analogues with the aspired characteristics: i.e. **high potency and selectivity for IRAP versus AP-N and especially AT_1 receptors, along with improved stability compared to Ang IV.** The most successful candidates will now be used for further *in vivo* work and functional *in vitro* assays. From the Ang IV analogues encompassing macrocyclic ring systems of different sizes obtained from Prof. Hallberg, it was demonstrated that disulfide cyclizations of Ang IV can deliver such ligands (Axén et al., 2006). Similarly, a recently synthesised analog containing two beta amino acids obtained from Prof. Tourwe displayed similar excellent characteristics.

In collaboration with Dr. S. Chakravarthy (Dept of Molecular Visual Plasticity, Netherlands Ophthalmic Research Institute, Amsterdam) we obtained the **'P40H1' hippocampal neuronal cell line**, established from a postnatal day 40 H-2Kb-tsA58 transgenic mouse hippocampus. P40H1 cells harbour the temperature-sensitive SV40 T-antigen, which makes them immortal at the permissive temperature of 34 °C. Preliminary [^{125}I]-Ang IV binding data revealed that this cell line displays a **high level of endogenous IRAP**. Currently we are investigating whether Ang IV (as was already shown for CHOK1 cells) is capable of increasing [^{3}H]-thymidine incorporation (to measure cell division) and/or triggering intracellular signalling pathways in these cells.

In adipocytes and muscle cells, it is well known that **IRAP co-localises with the insulin-dependent glucose transporter GLUT4** in specific intracellular vesicles. These vesicles move slowly to the cell surface. **Under the influence of insulin, this translocation proceeds ten times faster with significant glucose uptake as a result** (Bryant et al., 2002). Through this main mechanism insulin

regulates glucose homeostasis. In the brain, GLUT4 is expressed in the hippocampal formation, cortex, septum, hypothalamus, and cerebellum, and in most motor and motor-associated nuclei (El Messari et al., 1998). Interestingly, the distribution of GLUT4 strongly resembles that of IRAP (Fernando et al., 2005). Furthermore, it was demonstrated that GLUT4 and IRAP are co-localised in the same cells in selected nuclei of the brain, most notably the pyramidal cells in CA1-CA3 region of the **hippocampus** (Fernando et al., 2005). How the translocation occurs in the brain and whether insulin plays a key role, is not yet known. Studying this translocation and the role of IRAP might be important, because glucose was shown to have a positive effect on memory and learning in mice (Kopf et al., 2001). Additionally, by having more IRAP at the cell surface, signal transduction by Ang IV can be studied more easily. In view of this, we started to study these translocation mechanisms. Previous translocation studies of IRAP were hampered by a time-consuming detection of the enzyme. For example, cell surface IRAP was biotinylated and isolated by affinity chromatography, after which SDS-PAGE and western blotting was performed (Nakamura et al., 2000). Earlier indirect experiments also revealed that IRAP undergoes insulin-mediated translocation in the CHO-K1 cell model system despite the fact that they do not contain GLUT4 (Johnson et al., 1998, Lim et al., 2001). Based on our observation that CHO-K1 cells contain a large concentration of IRAP (Demaegdt et al., 2004) and on the methodology we already developed to specifically detect IRAP by radiolabelled Ang IV binding, we were able to measure the insulin-stimulated translocation of this protein in a more direct manner. This improved detection method relied on the inhibition of IRAP internalisation by phenylarsene oxide. With this method (Demaegdt et al., in preparation), we will study the translocation of IRAP to the cell surface in the previous mentioned hippocampal P40H1 cell line.

2. Investigation of the involvement and function of the IRAP/AT_4 system in vivo

Ang IV might mediate its memory-enhancing effects by preventing IRAP-mediated degradation of other neuropeptides involved in cognitive functioning, e.g. vasopressin, somatostatin and oxytocin. This would imply that inhibition of IRAP's catalytic activity by Ang IV may enhance the levels of these neuropeptides. Therefore, it is of major interest to **quantify neuropeptide release in brain dialysates following administration of Ang IV** or its analogues. Moreover, we are interested in **monitoring the** *in vivo* **levels of Ang IV itself** in baseline conditions, following pharmacological manipulation, during a memory task, and in pathophysiological conditions. Quantifying neuropeptides in microdialysates is challenging because low concentrations are expected and thus a selective and sensitive analysis technique is required, such as nano liquid chromatography (LC) electrospray (ESI) tandem mass spectrometry (MS/MS). Other LC detection methods are inadequate to achieve the required sensitivity and selectivity. With our ESI-MS detection, we observed reduced accuracy of the LC-MS/MS method for quantifying Ang IV in dialysates due to matrix effects and instability of Ang IV. We therefore investigated the use of an internal standard (IS), i.e. a structural analogue (Norleucine1-AngIV) or a stable isotopically-labeled (SIL) analogue, to improve our method. Linearity was improved when either of the proposed IS were applied. Only when using the SIL-IS, the repeatability of injection and the method's precision and accuracy was improved. Finally, the IS was able to correct for degradation of Ang IV in dialysates, prolonging the possible storage period of the samples. We concluded that the application of a SIL analogue is indispensable when quantifying Ang IV in dialysates using

nano LC-ESI-MS/MS detection (Lanckmans et al., submitted). We could not measure baseline levels of Ang IV although our limit of detection for Ang IV was about 10 pM (i.e. 10 attomol on column). However we succeeded to monitor alterations in Ang IV concentrations following Ang II administration (Lanckmans et al., in preparation).

Another long standing hypothesis states that Ang IV exerts its facilitatory effects on learning and memory via the AT_4 bindingsite, independently of AT_1 and AT_2 receptors. Moreover, facilitation of hippocampal cholinergic transmission is suggested to be involved in those memory enhancing effects. The role of the AT_1 receptor subtype in the dipsogenic effects and central pressor effects of the Ang IV precursors Ang II and Ang III is widely recognised (de Gasparo et al., 2000). Recent evidence indicated that AT_1 receptors are also involved in central pressor (Lochard et al., 2004) and peripheral vasoconstrictive effects of Ang IV (Li et al., 2006). **We therefore investigated the possible role of the AT_1 receptor subtype in effects of intracerebroventricular (i.c.v.) injected Ang IV and LVV-H7 on the drinking behaviour and hippocampal extracellular acetylcholine levels**. We provided direct *in vivo* evidence for differences in the biological effects elicited by the two 'AT4 receptor' ligands. Indeed, immediately after the i.c.v. injection of Ang IV, but not after the i.c.v. injection of LVV-H7, a drinking response was observed. The i.c.v. injection of the AT_1 receptor antagonist candesartan blocked the dipsogenic effect of Ang IV. Moreover, a tendency to increase followed by a significant sustained decrease of the extracellular hippocampal acetylcholine concentration was observed after i.c.v. administration of Ang IV but not of LVV-H7. Again i.c.v. injection of candesartan blocked this effect of Ang IV. These results suggest that cholinergic facilitation in the hippocampus is not involved in the spatial learning and memory enhancing effect of Ang IV and LVV-H7 (De Bundel et al., in preparation). Behavioural experiments in spatial mazes with AT_1 receptor antagonists may further elucidate the role of the observed attenuation of hippocampal acetylcholine levels in the learning and memory enhancing effects of Ang IV.

Concerning the exact nature of the IRAP/AT_4 system, we obtained in 2006 data suggesting that Ang IV exerts *in vivo* **effects either by inhibiting IRAP's enzymatic activity as well as by triggering certain cellular processes and thus acting as a receptor**. Indeed, Ang IV was anticonvulsant against limbic seizures and enhanced hippocampal monoamine levels, effects that were all blocked by a somatostatin sst2 receptor antagonist. Since somatostatin-14 is known as a substrate of IRAP and a known anticonvulsant, we hypothesized that the **inhibition of IRAP's catalytic activity by Ang IV enhanced the levels of somatostatin-14 in the brain** (Stragier et al., 2006). We also showed that local Ang IV administration enhances dopamine in rat striatum. Selective inhibition of AP-N, the enzyme necessary to metabolise Ang IV into smaller peptide fragments, by a compound named 7B (2(S)-benzyl-3-[hydroxyl(1'(R)-aminoethyl)phoshynyl]propanoyl-L-tyrosine), potentiated the Ang IV-evoked dopamine release, most probably due to a lengthening of the half-life of Ang IV. Non-selective inhibition of both IRAP and AP-N by 7B alone however failed to increase dopamine levels, demonstrating that the facilitation of striatal dopamine is not mediated via inhibition of IRAP's and/or AP-N's catalytic activity. Nevertheless, since Ang IV is known to bind to both aminopeptidases, we hypothesized that **in the striatum Ang IV may act as a agonist of a receptor dimer formed by IRAP and/or AP-N** (Stragier et al., 2007).

This year we have started collaboration with the Neuropeptides group of the Howard Florey Institute, University of Melbourne (Australia). Prof. Chai and Prof. Albiston of this group are the pioneers who discovered that the IRAP enzyme is the AT_4 bindingsite for Ang IV (Albiston et al., 2001). One of our PhD students, Dimitri De Bundel, is currently working at the University of Melbourne. The aim of this collaborative study is to **unravel whether AT_4/IRAP ligands may mediate some of their effects through modulation of hippocampal GLUT4 translocation to the plasma membrane and alteration of glucose uptake into the hippocampus of rodents**. We confirmed the decrease in extracellular hippocampal glucose levels, reflecting the higher metabolic need of the neurons, during the execution of a **spatial spontaneous alternation task in rats** (McNay et al., 2000) and are currently studying the effects of AT4/IRAP ligands on hippocampal glucose levels during the execution of this task. This study will elucidate the effects of AT4/IRAP on short term spatial memory, and a possible role of modulation of hippocampal glucose levels in the observed effects.

To quantify the memory-promoting effects of the ligands of our interest within this project, we now also possess a **validated set-up of the Morris water maze task**. The Morris water maze is the most widely used behavioral paradigm for **spatial learning and memory** in rats and mice. The animals learn to locate a platform which is hidden beneath the water surface of a circular pool by using distant cues. Since the Morris water maze task is especially valuable to restore learning in memory impairment models, we are currently investigating the effects of **continuous Ang IV administration** (via an implanted osmotic pump) in the **chronic amyloid-ß(1-42) model.** Our preliminary data show that amyloid ß(1-42)-treated rats learned the task efficiently but were unable to develop a clear spatial strategy, whereas Ang IV and amyloid ß (1-42)-treated rats were less efficient in learning the task but demonstrated a better spatial strategy.

Finally, in collaboration with Dr. S. Chai (Melbourne, Australia) we can, since recently, dispose over a strain of mice with a targeted deletion of the IRAP gene. This research group generated a mouse line in which the IRAP gene is flanked by LoxP sites (heterozygote IRAP-Floxed gene). Because these mice express the recombinant bacteriophage LoxP recombinase (cre), this enzyme will remove the floxed IRAP gene by splicing and recombination and generated heterozygous mice (knockout/wild-type). Accordingly we have setup the methodology for the genotyping of the offspring of these animals. For this purpose we extract and amplify the genomic DNA from the mouse-tails and we assess the expression of IRAP using primers that allow the detection of wild-type, heterozygote or homozygote IRAP knockout offspring. The birth of these **IRAP knockout mice** will lead to a major breakthrough in this project, because it will enable us to **prove that Ang IV indeed exerts its physiological** *in vivo* **effects** (e.g. the Ang IV-induced dopamine increase in striatum, monoamine facilitation in hippocampus, the anticonvulsant effects, the attenuation of hippocampal acetylcholine and GABA levels, the improvement of learning and memory function) **solely via the IRAP/AT_4 system.**

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III. PUBLICATION LIST OF THE EFAR RESEARCH GROUP ON THE IRAP ENZYME/AT4 RECEPTOR SYSTEM

1. International peer-reviewed publications on this specific topic before 2006

- (1) Vauqelin G, Michotte Y, Smolders S, Sarre S, Ebinger G, Dupont A and Vanderheyden P **(2002)** Cellular targets for angiotensin II fragments: pharmacological and molecular evidence. Review. *J Renin Angiotensin Aldosterone Syst.* **3(4):** 195-204. (scientific impact factor = 1.1)
- (2) Demaegdt H, Vanderheyden P, De Backer JP, Mosselmans S, Laeremans H, Le MT, Kersemans V, Michotte Y and Vauquelin G **(2004)** Endogenous cystinyl aminopeptidase in Chinese hamster ovary cells: characterization by [(125)I]Ang IV binding and catalytic activity. *Biochem Pharmacol* **68**: 885-892. (scientific impact factor = 3.4)
- (3) Demaegdt H, Laeremans H, De Backer JP, Mosselmans S, Le MT, Kersemans V, Michotte Y, Vauquelin G and Vanderheyden PM **(2004)** Synergistic modulation of cystinyl aminopeptidase by divalent cation chelators. *Biochem Pharmacol* **68**: 893-900. (scientific impact factor = 3.4)
- (4) Stragier B, Sarre S, Vanderheyden P, Vauquelin G, Fournie-Zalouski MC, Ebinger G and Michotte Y **(2004)** Metabolism of angiotensin II is required for its in vivo effect on dopamine release in the striatum of the rat. *J. Neurochem*. **90**: 1251-1257. (scientific impact factor = 4.8)
- (5) Laeremans H, Demaegdt H, De Backer JP, Le MT, Kersemans V, Michotte Y, Vauquelin G and Vanderheyden P (2005) Metal ion modulation of cystinyl aminopeptidase. *Biochem J* 390: 352-357. (scientific impact factor = 4.2)
- (6) Esteban V, Ruperez M, Sanchez-Lopez E, Rodriguez-Vita J, Lorenzo O, Demaegdt H, Vanderheyden P, Egido J and Ruiz-Ortega M **(2005)** Angiotensin IV activates the nuclear transcription factor-kappaB and related proinflammatory genes in vascular smooth muscle cells. *Circ. Res*. **96(9):** 965-973. (scientific impact factor = 9.4)
- (7) Stragier B, Hristova I, Sarre S, Ebinger G and Michotte Y (2005) In vivo characterization of the angiotensin-(1-7)-induced dopamine and gamma-aminobutyric acid release in the striatum of the rat. *Eur. J. Neurosci*. 22: 658-664. (scientific impact factor = 4.0)

2. International peer-reviewed publications on this topic in 2006

- (1) Demaegdt H, Lenaerts PJ, Swales J.,De Backer JP, Laeremans H, Le MT, Kersemans K, Vogel L, Michotte Y,Vanderheyden P and Vauquelin G (2006) AT_4 receptor ligand interaction with cystinyl aminopeptidase and aminopeptidase N: [^{125}I]Ang IV only binds to the cystinyl aminopeptidase apoenzyme. *Eur. J. Pharmacol.* 546: 19-27. (scientific impact factor = 2.5)
- (2) Vanderheyden P., Demaegdt H., Swales J., Lenaerts P.-J., Laeremans H., De Backer J.-P., Vogel L.K. and Vauquelin G **(2006)** Synergistic inhibition of the enzymatic activity of aminopeptidase N by divalent metal ion chelators. *Fund. Clin. Pharmacol*. **20**: 613-619. (scientific impact factor = 1.7)
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- (9) De Bundel D., Stragier B., Ceulemans A.-G., Smolders I., Sarre S., Ebinger G. and Michotte Y. **(2007)** The *in vivo* effects of angiotensin IV and IRAP ligands on the hippocampal acetylcholine levels. (in preparation)

3. PhD theses on this topic in 2006

- **Bart Stragier,** Characterization of the interaction between angiotensin fragments and the central neurotransmitter systems, academic year 2005-2006
- Promotor: Prof. Y. Michotte
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Phosphorylation of the amyloid precursor protein intracellular domain: regulation of the production of ß-amyloid peptide and transcriptional activity?

The coexistence of neurofibrillary tangles and senile plaques in the brain confirms the clinical diagnosis of Alzheimer's disease (AD) (1).

Intraneuronal neurofibrillary tangles are made of paired helical filaments containing the hyperphosphorylated microtubule associated protein tau (2). The amyloid core of extracellular senile plaques contains fibrils of amyloid beta peptide (Aβ) (3,4), which results from the cleavage of the amyloid precursor protein APP (5).

The most compelling evidence that Aβ is the causative agent of AD comes from observations on genetic mutations that cause familial forms of AD. Mutations of APP or presenilin genes alter the processing of APP (6), giving rise to increased production of Aβ, in particular of Aβ containing 42 amino acids (Aβ1-42), which is more prone to aggregation (7). These genetic data founded the amyloid cascade hypothesis (8), which implies that plaques should develop before tangles. However, neuropathological studies indicated that the initial development of tangles precedes the development of amyloid plaques by at least two decades (9). To reconcile these controversial observations, it has been proposed that the initial neurofibrillary changes are independent of Aβ (10), while this process is accelerated by the presence of Aβ, which can stimulate AD-like phosphorylation of tau in neuronal cultures (11).

For many years, the amyloid cascade hypothesis maintained that memory failure in AD derived from neuronal death induced by insoluble extracellular deposits of amyloid fibrils. Newer findings, however, demonstrate that accumulation of intraneuronal Aβ is neurotoxic (12,13).

A common alteration of the neuronal metabolism of APP and tau, leading to the production of both hyperphosphorylated tau and Aβ remains elusive.

Thanks to the Queen Elisabeth Medical Foundation, we studied the neuronal metabolism of APP and tau, following an increase of cytosolic calcium concentration resulting from the opening of voltage sensitive calcium channels in depolarizing conditions. In cultured neurons, membrane depolarization induced a calcium-mediated transient phosphorylation of APP and tau by the Cdk5 and GSK3 protein kinases, followed by a dephosphorylation of both proteins. Following transient phosphorylation of APP, intraneuronal Aβ1-42 accumulated and induced important neurotoxicity. This intraneuronal accumulation of Aβ1-42 was not observed following expression of the APPT668A mutant, indicating that phosphorylation of APP on Thr668 was indispensable. Thus, an increase of neuronal cytosolic calcium concentration may lead to both neuronal AD-like phosphorylation of tau and intraneuronal accumulation of Aβ, which induces neuronal death in AD.

These results were published in 2006 in the Journal of Biological Chemistry (J. Biol. Chem., Vol. 281, Issue 52, 39907-39914) in a paper entitled *«Calcium-mediated transient phosphorylation of tau and amyloid precursor protein followed by intraneuronal amyloid-beta accumulation.»* by Nathalie Pierrot, Susana Ferrao-Santos, Chrsitine Feyt, Marina Morel, Jean-Pierre Brion, and Jean-Noël Octave.

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Distinct contribution of parietal and frontal cortical areas to the control of finger and hand movements

Background

The aim of the present project was to determine the specific role of the frontal and parietal cortical areas involved in the control of skilled hand movements. Recent functional imaging studies have shown that precision grasping activates a large bilateral fronto-parietal network, though the actual contribution of the different areas composing this network remains largely unknown. Indeed, functional brain imaging studies do not allow us to make inferences about the causal relationship between the neural activity in a given region and the task under investigation. In contrast, transcranial magnetic stimulation (TMS) has proved very useful to overcome this limitation by producing, in healthy subjects, transient virtual lesions of a restricted brain area. Combined with a precise quantification of the deficits resulting from such virtual lesions (Duque et al., 2003), this technique permits to infer the contribution of the lesioned brain area to the task at hand. In order to determine exactly the coil position, we have used a TMS onto MRI coregistration technique developed in the laboratory (Noirhomme et al., 2004; Zosso et al., 2006) which allows us to normalize the individual coordinates of stimulation sites with respect to the Montreal Neurological Institute (MNI) brain atlas (see Davare et al., 2006).

1. Dissociating the role of ventral and dorsal premotor cortex in precision grasping

Small object manipulation is essential in numerous human activities though its neural bases are still largely unknown. Recent functional imaging studies have shown that precision grasping activates a large bilateral fronto-parietal network, including ventral (PMv) and dorsal (PMd) premotor areas. In order to dissociate the role of PMv and PMd in the control of hand and finger movements, we produced, by means of transcranial magnetic stimulation (TMS), transient virtual lesions of these two areas in both hemispheres, in healthy subjects performing a grip-lift task with their right, dominant, hand. We found that a virtual lesion of PMv specifically impaired the grasping component of these movements: a lesion of either the left or right PMv altered the correct positioning of fingers on the object, a prerequisite for an efficient grasping, whereas lesioning the left, contralateral, PMv disturbed the sequential recruitment of intrinsic hand muscles, all other movement parameters being unaffected by PMv lesions. On the other hand, we found that a virtual lesion of the left PMd impaired the proper coupling between the grasping and lifting phases, as evidenced by the TMS-induced delay in the recruitment of proximal muscles responsible for the lifting phase; lesioning the right PMd failed to affect dominant hand movements. Finally, an analysis of the time course of these effects allowed us to demonstrate the sequential involvement of PMv and PMd in movement preparation. These results provide the first compelling evidence for a neuronal dissociation between the different phases of precision grasping in human premotor cortex.

This paper has been published in *Journal of Neuroscience* (Davare et al., 2006) and has been commented by Chouinard in a paper also published in *Journal of Neuroscience* (Chouinard, 2006).

2. Role of the ipsilateral primary motor cortex in controlling the timing of hand muscle recruitment

The precise contribution of the ipsilateral primary motor cortex (iM1) to hand movements remains controversial. To address this issue, we elicited transient virtual lesions of iM1 by means of transcranial magnetic stimulation (TMS) in healthy subjects performing either a grip-lift task or a step-tracking task with their right dominant hand.

We found that, irrespective of the task, a virtual lesion of iM1 altered the timing of the muscle recruitment. In the grip-lift task, this led to a less coordinated sequence of grip and lift movements and, in the step-tracking task, to a perturbation of the movement trajectory. In the step-tracking task, we have demonstrated that disrupting iM1 activity may, depending on the TMS delay, either advance or delay the muscle recruitment.

The present study suggests that iM1 plays a critical role in hand movements by contributing to the setting of the muscle recruitment timing, most likely through either inhibitory or facilitatory transcallosal influences onto the contralateral M1 (cM1). iM1 would therefore contribute to shape precisely the muscular command originating from cM1.

This paper has been published in *Cerebral Cortex* (Davare et al., 2007).

3. Temporal dissociation of hand shaping and grip force scaling in the anterior intraparietal area (AIP)

In humans, both clinical and neuroimaging studies have evidenced the critical role of the posterior parietal cortex, and particularly of the anterior intraparietal area (AIP), in controlling skilled hand movements. However, the exact contribution of AIP to precision grasping remains much debated because quite a lot of different tasks have been shown to activate this area. To address this issue, we have used transcranial magnetic stimulation (TMS) to produce virtual lesions of the left and/or right AIP in healthy subjects performing a grip-lift task with either the right or left hand.

We found that virtual lesions of AIP had distinct consequences on precision grasping depending on its time of occurrence during movement preparation: lesions induced 270-220 ms before the fingers contacted the manipulandum affected specifically the hand shaping whereas lesions induced later, 170-120 ms before the contact time, altered only the grip force scaling. Another striking difference between the control of hand shaping and fingertip force by AIP is that, while a bilateral lesion was necessary to produce a deficit in hand posture, only a unilateral lesion of the left, but not right, AIP impaired the grip force scaling in either hand.

These findings suggest that both the left and right AIP are involved early in movement preparation and contribute equally to the visuomotor transformations required to adjust the hand posture to the object to grasp. Then, only the left AIP is implicated in the object weight representation used to scale the grip force anticipatively.

This paper is under revision in *Journal of Neuroscience* (Davare et al., under revision)

4. Contribution of hand motor circuits to counting

The finding that number processing activates a cortical network partly overlapping that recruited for hand movements has renewed interest in the relationship between number and finger representations. Further evidence about a possible link between fingers and numbers comes from developmental studies showing that finger movements play a crucial role in learning counting. However, increased activity in hand motor circuits during counting may unveil unspecific processes, such as shifting attention, reciting number names or matching items with a number name. To address this issue, we used transcranial magnetic stimulation to measure changes in corticospinal (CS) excitability during a counting task performed silently and using either numbers or letters of the alphabet to enumerate items. We found an increased CS excitability of hand muscles during the counting task, irrespective of the use of numbers or letters, whereas it was unchanged in arm and foot muscles. Control tasks allowed us to rule out a possible influence of attention allocation or covert speech on CS excitability increase of hand muscles during counting. The present results support a specific involvement of hand motor circuits in counting since no CS changes were found in arm and foot muscles during the same task. However, the contribution of hand motor areas is not exclusively related to number processing since an increase in CS excitability was also found when letters were used to enumerate items. This finding suggests that hand motor circuits are involved whenever items have to be put in correspondence with the elements of any ordered series.

This paper is in press in *Journal of Cognitive Neuroscience* (Andres et al., in Press).

5. Central representation of hand movements

We have also investigated hand movement representations in the central nervous system. Indeed, interaction with objects is critical in most daily activities and humans are able to access *a priori* knowledge of an object or of a tool to use it. Clinical studies on the ideomotor apraxia, a deficit characterized by an inability to recall the appropriate hand posture to use tools, have suggested that the left posterior parietal cortex (PPC) may store a repertoire of gestures tool use. Moreover, several imaging studies have suggested that, within the PPC, the critical region for hand movement representations may be the left inferior parietal lobule (see Fadiga et al., 2005 for review). To address this issue experimentally, we have compared, in healthy subjects, the consequences of virtual lesions of Brodmann area 7 (BA7) and of the supramarginal gyrus (SMG) on a hand rotation task and on a control task using letters. We have found that only a virtual lesion of the SMG, as induced with TMS, affected the reaction time in the hand rotation task performance was only altered by a BA7 lesion (Pelgrims et al., *submitted*).

6. Reorganisation of the corticospinal system (CS) following congenital lesions.

Finally have also investigated the reorganisation of the corticospinal projections in patients suffering from a congenital hemiplegia (Vandermeeren et al., *submitted*). We have also quantified as precisely as possible the correlation between the dysgenesis of the corticospinal tract in those patients and the functional recovery; CS tract dysgenesis was quantified by means of the DTI technique We confirmed our previous finding (Duque et al., 2003) and we further showed that the quantification of the CS dysgenesis by means of DTI is far more precise than with conventional measurement (Bleyenheuft et al., *submitted*).

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Characterization of the role of G protein-coupled receptors in the central nervous system by using genetically invalidated mouse models.

1. Overview

The activities of the group are centered onto G protein-coupled receptors, which represent the largest family among membrane receptors. They all share a common structural organization with seven transmembrane segments, and a common way of modulating cell function through a family of heterotrimeric G proteins. About 180 G protein-coupled receptor types and subtypes have been functionally characterized to date in mammalian species, and about 100 orphan receptors are presently available in the literature or the databases. Orphan receptors potentially constitute elements of unknown communication pathways in various systems. The general aim of this program is to identify novel receptors playing a role in brain physiology. The characterization of the function of known receptors or recently characterized receptors, by using, among other approaches, knockout models, has also been pursued.

2. The glucocorticoid-induced receptor (GIR)

Glucocorticoid-induced receptor (GIR) is an orphan GPCR with predominant expression in brain and thymus. High levels of GIR expression have been described in limbic forebrain and hypothalamic regions of the brain of mouse, rat and human, suggesting a role for GIR in memory, cognition, stress, reward or the control of emotion. We have generated over the last years a knock-out model for GIR in which part of the coding region is replaced by a tau-LacZ reporter gene.

Using this reporter gene, we confirmed the high expression of GIR in scattered large striatal neurons coexpressing choline acetyl-transferase, a specific marker for cholinergic neurons. Strong labelling was also observed in neurons of the olfactory bulb, the olfactory tubercle, the thalamus, and less abundantly in the piriform cortex and hippocampus. The knockout mice were tested in a number of behavioural settings. The mice displayed hyperlocomotion in the open field and were prone to anxiety. Motor coordination was affected, particularly in old mice, as shown in the rotarod and strength grip tests. In situ hybridization has shown reduced expression of the proenkephalin gene and overexpression of substance P and prodynorphin genes, suggesting increased activity of dopamine D_1-expressing neurons and reduced activity of D2-expressing neurons, while the number of D_1 and D_2 binding sites in the striatum appeared unchanged. Microarray analysis of the striatum has revealed overexpression in knockout mice of a number of genes also upregulated following acute or chronic cocaine treatment (c-fos, egr1, egr2, PP-1, Na/K ATPase). Cocaine administration resulted in stimulated locomotion in both genotypes, but more efficiently in KO animals. The treatment with D_1 and D_2 agonists or antagonists also affected the KO and wild-type mice differently. We are now investigating the mechanisms underlying the apparent hyperactivity of the dopaminerguic system in the striatum of GIR knockout mice (Laurent et al. unpublished).

3. Further characterization of a mouse knock-out model for the A_{2a} adenosine receptor

Adenosine is released from metabolically active cells or generated extracellularly. It is a potent biological mediator that contributes to the protection of cells and tissues during stress conditions such as ischaemia. We had previously generated a knockout model for the A_{2a} receptor (Ledent et al. Nature 388: 674-678, 1997). Additional experiments were made in collaboration with different groups, in order to delineate further the role of adenosine receptors in various aspects of physiology.

Dopamine and adenosine receptors are known to share a considerable overlap in their regional distribution, being especially rich in the basal ganglia. Dopamine and adenosine receptors have been demonstrated to exhibit a parallel distribution on certain neuronal populations, and even when not directly co-localized, relationships (both antagonistic and synergistic) have been described. We investigated dopaminergic and purinergic systems in mice with ablations of individual dopamine or adenosine receptors. In situ hybridization histochemistry and autoradiography was used to examine the level of mRNA and protein expression of specific receptors and transporters in dopaminergic pathways. Expression of the mRNA encoding the dopamine D_2 receptor was elevated in the caudate putamen of D_1, D_3 and A_{2A} receptor knockout mice; this was mirrored by an increase in D_2 receptor protein in D_1 and D_3 receptor knockout mice, but not in A_{2A} knockout mice. Dopamine D_1 receptor binding was decreased in the caudate putamen, nucleus accumbens, olfactory tubercle and ventral pallidum of D_2 receptor knockout mice. In substantia nigra pars compacta, dopamine transporter mRNA expression was dramatically decreased in D_3 receptor knockout mice, but elevated in A_{2A} receptor knockout mice. All dopamine receptor knockout mice examined exhibited increased A_{2A} receptor binding in the caudate putamen, nucleus accumbens and olfactory tubercle. These data are consistent with the existence of functional interactions between dopaminergic and purinergic systems in these reward and motor-related brain regions (Short et al. 2006).

A_{2A} adenosine and D_2 dopamine receptors are colocalized in the same neurons in discrete brain areas, and the dopaminergic transmission plays a crucial role in the addictive properties of drugs of abuse, such as cocaine. We have investigated the specific role of A_{2A} adenosine receptors in cocaine-induced behavioral responses related to its addictive properties. Acute cocaine induced a similar increase of locomotor activity in mice lacking A_{2A} adenosine receptors and wild-type littermates. Cocaine-induced locomotor sensitization and conditioned place preference were also maintained in A_{2A} knockout mice. Nevertheless, knockout mice showed a lower rate of cocaine self-administration than wild-type mice in fixed ratio schedules of reinforcement. Moreover, a reduction in the maximal effort to obtain a cocaine infusion was found in A_{2A} knockout mice under a progressive ratio schedule. In addition, a vertical shift of the cocaine dose-response curve was observed in mice lacking A_{2A} receptors. These results demonstrate that A_{2A} adenosine receptors play an important role in cocaine addictive properties, and that these receptors are required to develop the addictive effects of this drug (Soria et al. 2006).

The non-selective A_{2A} antagonist caffeine has been reported to modify nicotine-induced locomotor and reinforcing effects. We investigated the specific role of A_{2A} receptors in the behavioural responses induced by nicotine by using knockout mice. Acute nicotine administration induced

a similar decrease of locomotor activity in A_{2A} knockout mice and wild-type littermates. Acute antinociceptive responses elicited by nicotine in the tail-immersion and hot-plate tests were unaffected in these mutant mice. The rewarding properties of nicotine were then investigated using the place-conditioning paradigm. Nicotine-induced conditioned place preference was suppressed in A_{2A} knockout mice. Accordingly, in vivo microdialysis studies revealed that the extracellular levels of dopamine in the nucleus accumbens were not increased after nicotine administration in mutant mice. Wild-type and A_{2A} knockout mice were trained in conditioned taste aversion procedure in which drinking a saccharin or saline solution was paired with nicotine or saline injections. A similar reduction in the intake of nicotine-paired solution in this paradigm was obtained in both genotypes. Finally, the administration of the nicotinic antagonist mecamylamine in nicotine-dependent mice precipitated a similar withdrawal syndrome in both genotypes. Together, these results identify A_{2A} adenosine receptors as an important factor that contributes to the rewarding properties of nicotine (Castane et al. 2006).

We also investigated the involvement of adenosine receptors in the interaction between paracetamol and caffeine in mice, using the adenosine A_{2A} receptor antagonist SCH58261 and the adenosine A_{2B} receptor antagonist PSB1115, in the tail immersion and hot-plate tests. Paracetamol was antinociceptive in both tests, but, in contrast to previous studies, caffeine was pronociceptive in the tail immersion test, and reduced the effects of paracetamol in both tests. SCH58261 was antinociceptive in both tests and in its presence paracetamol had no further effect. PSB1115 had little effect alone but potentiated the effect of paracetamol in the hot-plate test and abolished it in the tail immersion test. These results suggest that adenosine A_{2B} receptors may be involved in the action of paracetamol in a pathway-dependent manner, and also support the existence of pronociceptive adenosine A_{2A} receptors (Godfrey et al. 2006).

4. Characterization of a mouse knock-out model for the central cannabinoid receptor CB_1

We had previously generated a knockout model for the CB_1 receptor, the central receptor for the active compounds of *Cannabis*, and for the endogenous cannabinoid anandamide (Ledent et al. Science 285 : 401-404, 1999). This model was further tested in collaboration with a number of groups.

We studied the effects of cannabinoids on contextual conditioned fear responses. CB_1 knockout and wild-type (CD1) mice were exposed to a brief session of electric shocks, and their behavior was studied in the same context 24 h later. In wild-type mice, shock exposure increased freezing and resting, and decreased locomotion and exploration. The genetic disruption of the CB_1 receptor abolished the conditioned fear response. The CB1 antagonist AM-251 reduced the peak of the conditioned fear response when applied 30 min before behavioral testing (i.e. 24 h after shocks) in CD1 (wild-type) mice. The cannabinoid agonist WIN-55,212-2 markedly increased the conditioned fear response in CD1 mice, the effect of which was potently antagonized by AM-251. Thus, cannabinoid receptor activation appears to strongly promote the expression of contextual conditioned fear. In earlier experiments, cannabinoids did not interfere with the expression of cue-induced conditioned fear but strongly promoted its extinction. Considering the primordial role of the amygdala in simple associative learning (e.g. in cue-induced fear) and the role of the

hippocampus in learning more complex stimulus relationships (e.g. in contextual fear), the present and earlier findings suggest that cannabinoid signaling plays different roles in the two structures (Mikics et al. 2006).

Endocannabinoids play central roles in retrograde signaling at a wide variety of synapses throughout the CNS. Although several molecular components of the endocannabinoid system have been identified recently, their precise location and contribution to retrograde synaptic signaling is essentially unknown. By using two independent riboprobes, we showed that principal cell populations of the hippocampus express high levels of diacylglycerol lipase alpha (DGL-alpha), the enzyme involved in generation of the endocannabinoid 2-arachidonoyl-glycerol (2-AG). Immunostaining with two independent antibodies against DGL-alpha revealed that this lipase was concentrated in heads of dendritic spines throughout the hippocampal formation. Furthermore, quantification of high-resolution immunoelectron microscopic data showed that this enzyme was highly compartmentalized into a wide perisynaptic annulus around the postsynaptic density of axospinous contacts but did not occur intrasynaptically. On the opposite side of the synapse, the axon terminals forming these excitatory contacts were found to be equipped with presynaptic CB_1 cannabinoid receptors. This precise anatomical positioning suggests that 2-AG produced by DGL-alpha on spine heads may be involved in retrograde synaptic signaling at glutamatergic synapses, whereas CB_1 receptors located on the afferent terminals are in an ideal position to bind 2-AG and thereby adjust presynaptic glutamate release as a function of postsynaptic activity. We propose that this molecular composition of the endocannabinoid system may be a general feature of most glutamatergic synapses throughout the brain and may contribute to homosynaptic plasticity of excitatory synapses and to heterosynaptic plasticity between excitatory and inhibitory contacts (Katona et al. 2006).

Cannabinoids, acting through the CB_1 cannabinoid receptor, protect the brain against ischemia and related forms of injury. This may involve inhibiting the neurotoxicity of endogenous excitatory amino acids and downstream effectors, such as nitric oxide (NO). Cannabinoids also stimulate neurogenesis in the adult brain through activation of CB_1R. Because NO has been implicated in neurogenesis, we investigated whether cannabinoid-induced neurogenesis, like cannabinoid neuroprotection, might be mediated through alterations in NO production. W measured neurogenesis in dentate gyrus (DG) and subventricular zone (SVZ) of CB_1-KO and wild-type mice, some of whom were treated with the cannabinoid agonist Win 55212-2 or the NO synthase inhibitor 7-nitroindazole (7-NI). NOS activity was increased by approximately 25%, whereas bromodeoxyuridine (BrdU) labeling of newborn cells in DG and SVZ was reduced by approximately 50% in CB_1-KO compared with wild-type mice. 7-NI increased BrdU labeling in both DG and SVZ and to a greater extent in CB_1-KO than in wild-type mice. In addition, Win 55212-2 and 7-NI enhanced BrdU incorporation into neuron-enriched cerebral cortical cultures to a similar maximal extent and in nonadditive fashion, consistent with a shared mechanism of action. Double-label confocal microscopy showed coexpression of BrdU and the neuronal lineage marker doublecortin (Dcx) in DG and SVZ of untreated and 7-NI-treated CB_1-KO mice, and 7-NI increased the number of Dcx- and BrdU/Dcx-immunoreactive cells in SVZ and DG. Thus, cannabinoids appear to stimulate adult neurogenesis by opposing the antineurogenic effect of NO (Kim et al. 2006).

5. Huntington disease model

There is presenytly a controversy in the litterature regarding the effects of minocycline in the R6/2 transgenic model of Huntington's disease. We have therefore tested this tetracycline in another model, the N171-82Q strain. Ten miligrams per kilogram minocycline was given daily from the age of 2 mo, corresponding to an early symptomatic stage of Huntington's disease. We did not observe improvement in survival, weight loss, or motor function in treated transgenic mice. In addition, minocycline failed to mitigate the ventricle enlargement as well as the striatal and cortical atrophies observed in the transgenic line. Using high-performance liquid chromatography, it was observed that minocycline was similarly present in the plasma and the brain of both wild-type and N171-82Q mice following 14 daily injections. Using Western blot, we showed that the increased expression of procaspase-1 induced by the transgene in the cortex was significantly reduced by the antibiotic. These data support that despite minocycline crossing the blood-brain barrier and inhibiting procaspase-1 expression, it did not provide protection in this Huntington's disease model. The present data do not support minocycline as a beneficial drug for Huntington's disease (Mievis et al. 2006).

6. Dimerization of GPCRs and functional consequences

It became clear over the recent years that most, if not all, G protein-coupled receptors (GPCR) are able to form dimers or higher order oligomers. Chemokine receptors make no exception to this new rule and both homo- and heterodimerization were demonstrated for CC and CXC receptors. Using CCR5 and CCR2 as models, we previously demonstrated that these chemokine receptors form homo- and heterodimers and that dimers can only bind a single chemokine molecule with high affinity. We have now provided evidence from bioluminescence resonance energy transfer experiments that stimulation by chemokines does not influence the CCR2/CCR5 heterodimerization status. In addition, we show that the rate of radioligand dissociation from one unit of the heterodimer in "infinite" tracer dilution conditions is strongly increased in the presence of an unlabeled chemokine ligand of the other unit. These results demonstrate unambiguously that the interaction between heterodimer units is of allosteric nature. Agonists, but also some monoclonal antibodies, could promote such negative binding cooperativity, indicating that this phenomenon does not require the full conformational change associated with receptor activation. Finally, we show that G protein coupling is required for high-affinity binding of MIP-1β (CCL4) to CCR5 and that the dissociation from G proteins, after incubation with Gpp(NH)p, promotes the release of prebound radiolabeled chemokines with kinetics similar to those measured after the addition of an excess of unlabeled chemokines. These observations suggest that the association with G proteins probably participates in the negative cooperativity observed between receptor monomers. Such negative cooperativity within homo- and heterodimers can likely be extended to other clases of G protein-coupled receptors, including in the central nervous system, and will have major implications in the pharmacology of these receptors in vivo and in the physiopathology of the diseases in which they are associated (Springael et al. 2006).

7. P2Y receptors

Extracellular ATP is known to affect the maturation of monocyte-derived dendritic cells mainly by regulation of cytokines and costimulatory molecules. We have shown that adenine nucleotides inhibit the release of MCP-1 (CCL2) and MIP-1α (CCL3) by human monocyte-derived dendritic cells through the P2Y11 and P2Y1 purinergic receptors, resulting in a strongly reduced capacity to attract other leucocyte populations (Horckmans et al. 2006).

8. Chemokine receptors

The regulation by various cytokines of CXCL10 expression was studied in human fibroblasts and microvascular endothelial cells. IL-1β and TNF-α synergized with IFN-α,β or γ for CXCL10 induction. High synovial CXCL10 concentrations were found in autoimmune arthritis (ankylosing spondylitis, psoriatic arthritis and rheumatoid arthritis) (Proost et al. 2006).

We studied the role of CCR5 and its ligands in a model of acute pancreatitis (ip injection of cerulein) in mice. Induction of AP resulted in an early increase of pancreatic CCL2, CCL3, and CCL4, followed by recruitment of $CCR5^+$ cells. In knock out mice, lack of CCR5 exacerbated pancreatitis and led to increased levels of CC chemokines and a more pronounced pancreatic inflammatory infiltrate. These results suggest that CCR5-expressing cells modulate the severity of acute pancreatitis (Moreno et al. 2006).

Human T-cell leukemia virus type-1 (HTLV-1) is associated with adult T-cell leukemia (ATL) and neurological syndromes. HTLV-1 encodes the oncoprotein Tax-1 that modulates viral and cellular gene expression leading to T-cell transformation. We reported an interaction between HTLV-I Tax oncoprotein and the G protein beta subunit. The G protein beta subunit inhibits Tax-mediated viral transcription, while Tax-1 perturbs G protein beta subcellular localization. Functional evidence for these observations was obtained using conditional Tax-1-expressing transformed T-lymphocytes, where Tax expression correlated with activation of the SDF-1/CXCR4 axis. HTLV-1 has therefore developed a strategy based on the activation of the SDF-1/CXCR4 axis in the infected cell (Twizere et al. 2006).

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LONG-TERM MEMORY IN MOUSE HIPPOCAMPAL SLICES

Background

For neurocomputing theorists, every memory is encoded in a neuronal network thanks to a change in the distribution of its synaptic weights. Among the possible biological mechanisms, long-term potentiation (LTP) is the favored candidate to be at the basis of memory storage because this activity-induced increase in synaptic strength is (1) durable and (2) input-selective (restricted to the activated synapses). This phenomenon can be studied on an in vitro preparation. In a thin hippocampal slice (0.5 mm thick) maintained artificially alive it is possible to induce an LTP of the strength of the synapses by appropriate electrical stimulation of the presynaptic fibers. Two temporal phases of LTP can be induced and explored. A short-lasting LTP (S-LTP), which lasts 1-2 h, can be triggered by application of a single train of high frequency stimulation (HFS). S-LTP has been intensively studied and the underlying mechanisms are relatively well known. They consist of incorporation of extra chemoreceptors in the postsynaptic membrane and phosphorylation of proteins. A more durable LTP can also be elicited in hippocampal slices. This long-lasting LTP (L-LTP) is known for long but the mechanisms underlying its late phase are very poorly understood. Classically, L-LTP has three attributes. The first is of course its "lateness", the second is the fact that its induction necessitates multiple trains of tetanic stimuli and the third is that its induction requires a change in gene expression and protein synthesis. The dependence of L-LTP on protein synthesis poses a series of questions. (1) What is the identity of the proteins involved in L-LTP induction? (2) What are the signaling cascades allowing to the Ca^{++} entry to trigger protein synthesis? (3) What is the mechanism underlying input selectivity in L-LTP? How are the products of the activated genes (located in the nucleus which rules the whole neuron) headed to only the activated synapses. Frey and Morris have proposed in 1997 that the activation of a synapse tags it in such a way that it becomes enable to capture the products of protein synthesis. This raises two crucial questions: (1) What is the signaling cascade between synaptic activity and "tag" formation? (2) What is the nature of the tag? Our research is devoted to the mechanisms related to the forementioned problems. During this year, our contribution to these questions raised by L-LTP was the following

I. Does L-LTP require repetitive activity of the involved synapses for its induction? (second attribute of L-LTP)

It is nearly a dogma that induction of L-LTP requires application of multiple trains of high frequency stimulation (3 trains at 10 min interval or 4 trains at 5 min interval). This suggests that a stimulation pattern equivalent to multiple learning sessions is required for L-LTP induction. We demonstrated this not to be the case.

1. Classically, LTP is studied on hippocampal slices maintained "in interface" as well as during recovery from the trauma caused by slicing as during recordings. In the interface slice preparation, slices are partially submerged in artificial cerebro-spinal fluid (ACSF) and the top surface of the slice is exposed to a humidified atmosphere of 95% O_2 and 5% CO_2. In this case the slice gets oxygen that is diffused through the very thin film of liquid covering the

slice. We found that if the slice was allowed to recover in submersion (in the submerged-slice preparation, slices are completely submerged in oxygenated ACSF) an L-LTP can be easily induced by a single train of HFS.
This result has been published in Learning and Memory (Capron et al. 2006).

2. On a preparation where recovery and recordings occurred "in interface", we found that when a single train of HFS was applied 45 min after application of the SK channel blocker apamin, it induced an L-LTP of several hours, instead of an S-LTP of 1-2 h. We found that this metaplastic effect of apamin was crucially dependent on the NO-synthase pathway.
These results have been submitted to Learning and Memory (Ris et al. 2007).

II. Signaling cascades involved in triggering protein synthesis in L-LTP

At least three signaling cascades can make the link between the increase in Ca^{++} concentration and the triggering of protein synthesis. First cascade. Ca^{++} activates adenylate cyclase. The resulting increase in AMPc concentration stimulates protein kinase A (PKA). As a result, PKA dissociates into its two catalytic domains and its two regulatory domains. The dissociated catalytic domain of PKA translocates into the nucleus and via CREB phosphorylation, triggers protein synthesis. A second cascade involved mitogen-activated protein kinase (MAPK). Ca^{++} activates MAPK-KK (kinase of kinase of kinase) which, in turn, activates MAPK-K which, in turn, phosphorylates MAPK. Phosphorylated MAPK translocates into the nucleus and also via CREB phosphorylation, triggers protein synthesis. The third cascade involves CaMKIV (calcium calmodulin kinase number four), an enzyme constitutionally located in the nucleus. This enzyme, whose activity leads to protein synthesis, is activated by Ca^{++} associated with calmodulin.

However, CaMKIV is one of the two main targets of CaMKK (kinase of members of the CaMK family) which has two isoforms (CaMKKα and CaMKKβ). In collaboration with Prof. Giese at the University College of London who made CaMKK (α or β) knocked out mice, we explored whether CaMKK (α and β) played a role in the late phase of L-LTP. Giese's team found deficits in certain types of learning which were different in male and female mice. Consequently, we tested slices from female mice and male mice separately. We found no defect of L-LTP both in the male and in the female CaMKKα hippomorphic mice.

This result has been published in Molecular and Cellular Biology (Mizuno et al. 2006) and has been featured by Science SKTE (N.R. Gough, Males Afraid, Females Brave. Sci. STKE 2006, tw400 (2006).

By contrast, we found that CaMKKβ had a male-specific role in L-LTP. The late phase of L-LTP was impaired in slices from male CaMKKβ knocked out mice but not in female mutants.

This result is in press in Neuroscience (Mizuno et al. 2007)

III. Loss of input selectivity of L-LTP with aging

Memory shows age-related decline. According to the current prevailing theoretical model, encoding of memories relies on modifications in the strength of the synapses connecting the different cells within a neuronal network. The selective increases in synaptic weight are thought to be biologically implemented by long-term potentiation. During this year, we explored the possibility that input specificity of LTP could vanish with aging and hence hinder memory.

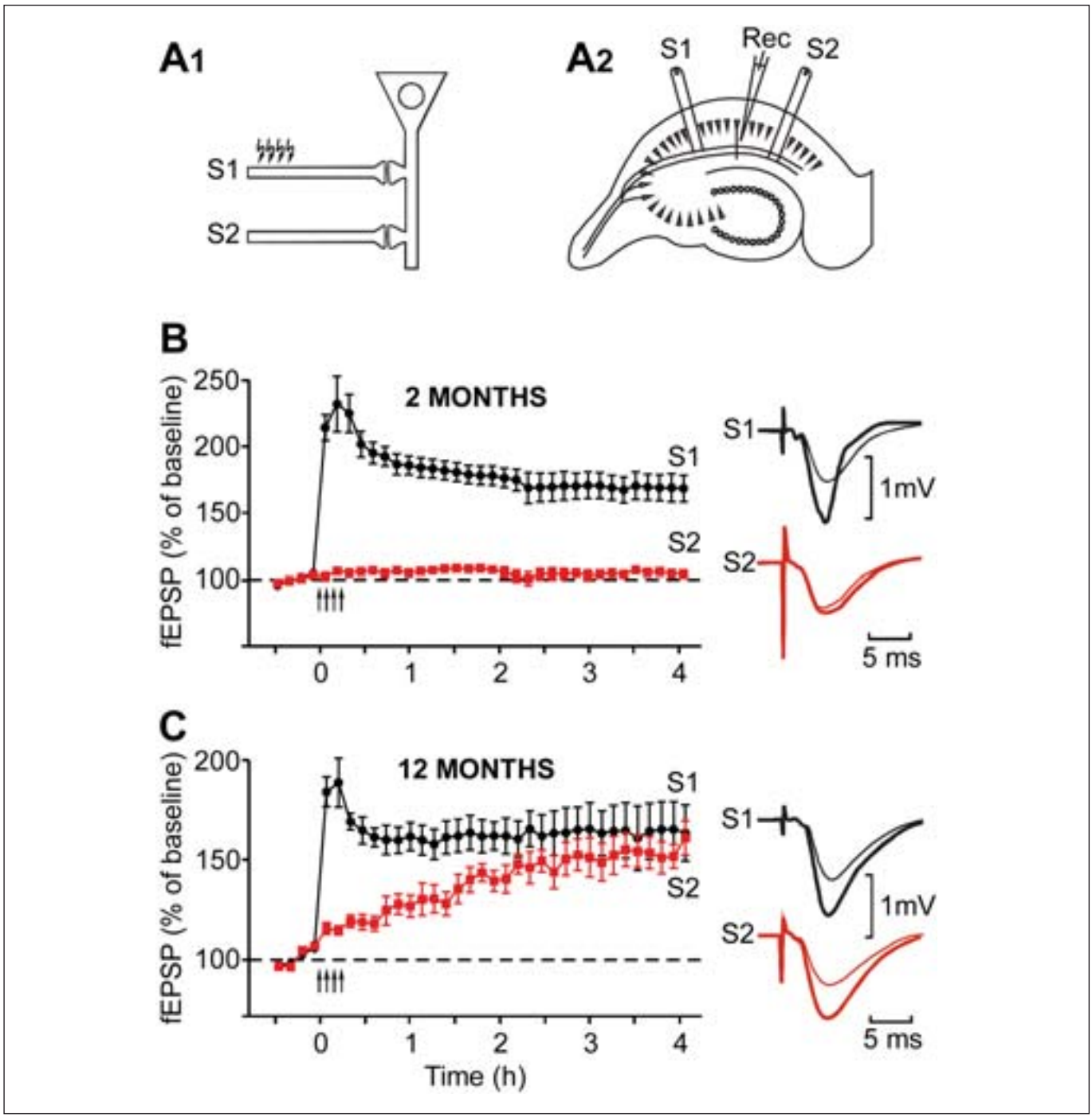

Figure 1. Synapse specificity of long-term potentiation breaks down with aging.

Two groups of mice (2 and 12 months old) were compared. Two distinct bundles of Schaffer collaterals were stimulated (S1 and S2) and the responses that both of them evoked in a same group of CA1 pyramidal neurons were recorded (Fig. 1 A1 and A2). In young mice, we confirmed the well-established input selectivity of LTP (Fig. 1B). When 4 trains were applied through S1, an LTP developed in the synapses tested via S1, while the strength of the synapses tested via S2 remained unchanged. This situation was totally different in mice aged 12 months (Fig. 1C). In this group of mice, application of 4 trains of stimulation through S1 triggered not only an LTP in the synapses tested via S1, but also a progressively developing increase in the strenght of the synapses tested via S2. We further demonstrated that this phenomenon was suppressed by blocking either L-type Ca^{++} channels or Ca^{++}-induced Ca^{++} release, both well known to become dysregulated with aging.

Our results not only demonstrate that synapse specificity of LTP breaks down with aging but also strongly suggest that the induction of the formation of a "tag" by synaptic activity is mediated by Ca^{++}.

These results are in press in Learning and Memory (Ris and Godaux, 2007).

IV. Elaboration of a technique of identification of a modified protein in brain microsamples

As one of our aims is to identify proteins involved in L-LTP induction and in L-LTP selectivity (nature of the tag), a prerequisite was to elaborate a technique capable to identify a protein modified as a result of synaptic activity.

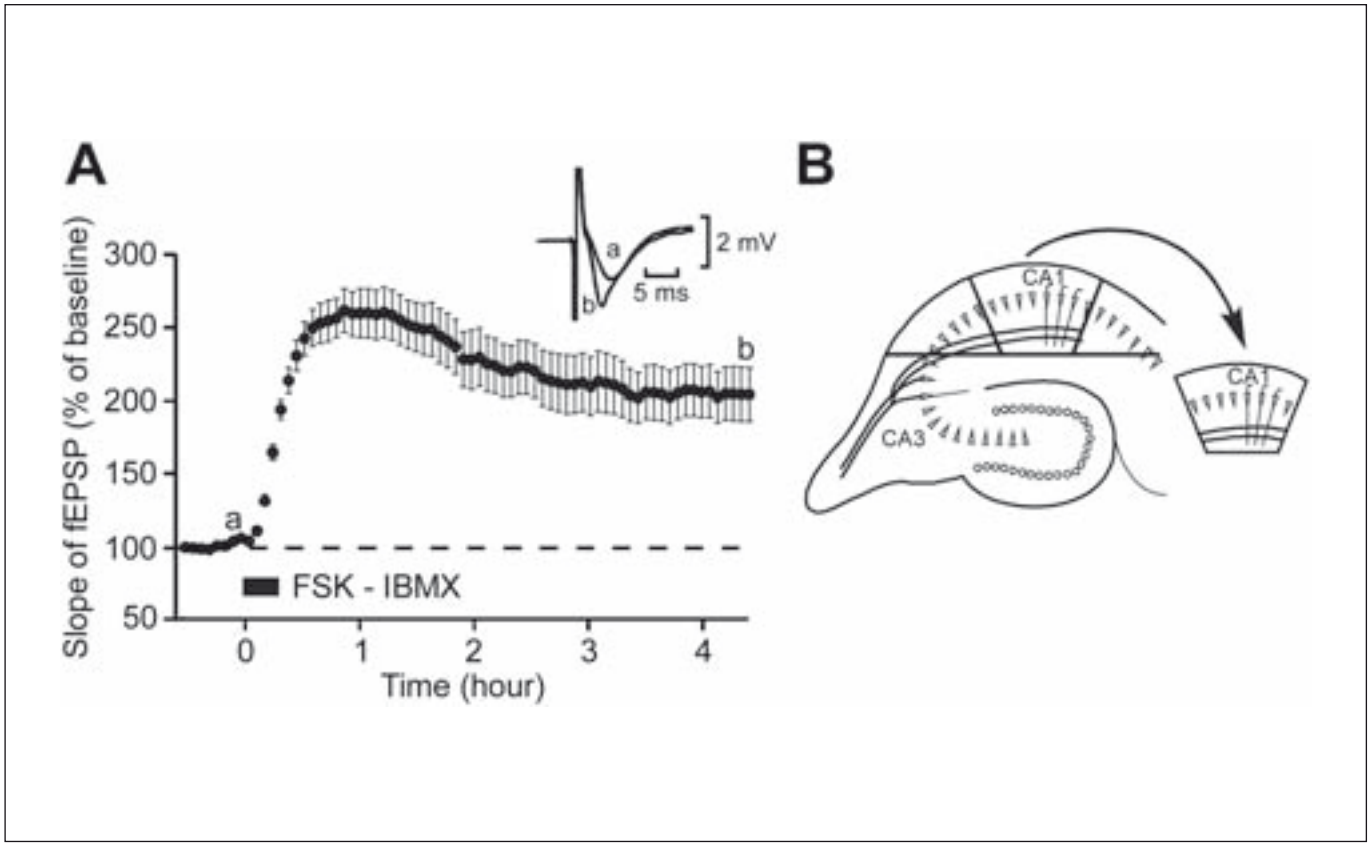

Figure 2. Chemical L-LTP and biochemical analysis of microsamples from hippocampal slices.

With such an aim in mind, an L-LTP was induced chemically (in order to cause a change in a great number of synapses) by applying forskolin, an activator of adenylate cyclase and IBMX, an inhibitor of phosphodiesterase (Fig. 2A). The CA1 region was removed from each hippocampal slice using a razor blade under a dissecting microscope (Fig. 2B). Each CA1 sample intended to be submitted to further biochemical analysis was obtained by pooling the CA1 regions taken from six individual slices and provided 50 µg of protein. These proteins were then separated by two-dimension electrophoresis (2-DE). A modification of a protein occurring during L-LTP could then be spotted on these 2-DE maps. For instance, using anti-phosphotyrosine antibody, we detected a protein that was strongly phosphorylated on tyrosine during L-LTP. The spots corresponding to that protein could then be punched and further identified using matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF MS). In the case used as a trial, the phosphorylated protein spotted was found to be rabphilin, a protein present in presynaptic terminals.

These results are in press in Neuroscience Letters (Capron et al. 2007).

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Roles of the direct and indirect pathways in functions and disorders of the basal ganglia.

I. Direct and Indirect pathways in basal ganglia functions and disorders

Gene targeting of the striatal neuronal subpopulations to investigate the roles of indirect and direct pathways of basal ganglia

Our aim was to generate transgenic mice allowing the study of the specific roles of striatopallidal or striatonigral neurons. We have obtained mice strains expressing the Cre recombinase under the control of the A_{2A} receptor promoter inserted in a BAC (bacterial artificial chromosome) to direct gene exp ression specifically in striatopallidal neurons. These lines have been crossed with a reporter strain (Rosa26-LacZ) in order to determine whether they selectively expressed Cre in these striatopallidal neurons. Co-localisation experiments using anti-enkephalin antibody (Enk) and LacZ activity detection showed that >80% of the LacZ neurons are also Enk+. Retrograde labelling of striatonigral neurons combined with the immunodetection of β-galactosidase showed that striatonigral are LacZ-negative. The same lines of Cre mice have been also crossed with three different lines of reporter mice allowing the expression of GFP or analogues proteins. One of these, the reporter mice expressing Z/EG under the control of the pCAGGS promoter (Novak et al., 2000), demonstrated the expression of eGFP restricted in a subpopulation of striatal neurons which were never identified as striatonigral by retrograde labelling and who co-expressed D2 and A_{2A} receptors as demonstrated by single cell RT-PCR. Therefore, altogether, these results demonstrated that we have generated strains of transgenic mice with a specific Cre expression in the striatopallidal neurons (see figure 1) (de Kerchove d'Exaerde et al., 2006). In the same line of works, we have collaborated to the construction and generation of another line of mice expressing specifically Cre recombinase in the striatum (Lemberger et al., 2006).

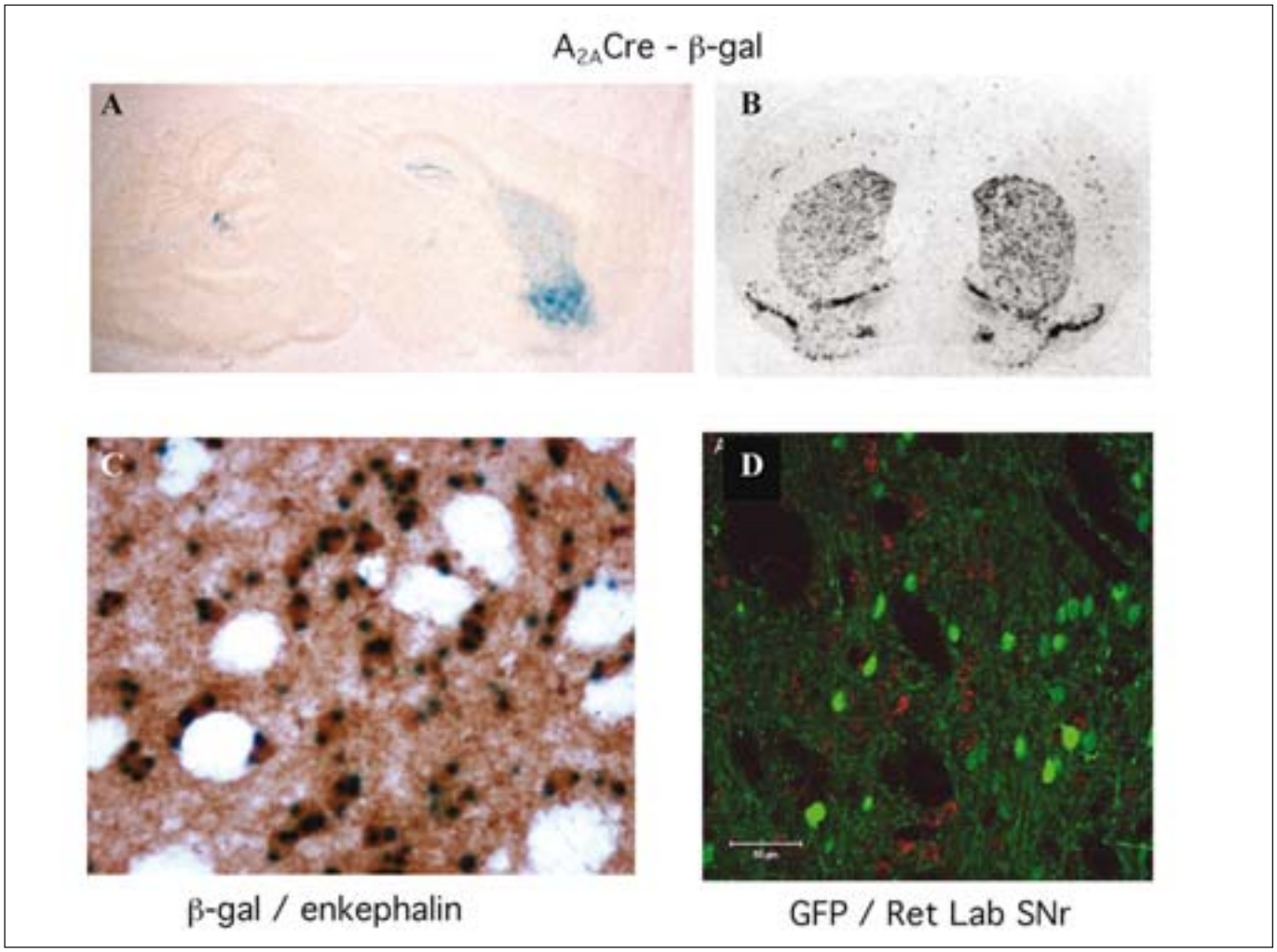

Figure 1: Reporter mice (Rosa26-lox-Stop-lox-LacZ (A,B,C) or pCAGGS-lox-STOP-lox-EGFP (D)) crossed with A_{2A}r-Cre mice were used to localize Cre-mediated recombination.

(A,B) β-galactosidase histochemistry (blue) shows a restricted staining in the striatum in parasaggital (A) and coronal sections (B). Co-labeling with enkephalin-immunocytochemistry (brown) (C) or excluded labeling of GFP (green) with the retrograde labeling of striatonigral neurons (red) (D) demonstrates the restricted recombination in striatopallidal neurons.

The striatopallidal-GFP mice has been used as a tool to specifically isolate this population of striatal neurons by using dissociation and sorting by FACS and to specifically record them by using the patch clamp technique. We succeed in isolating green (striatopallidal) and red (striatonigral) neurons from individual mice with a very good rate of enrichment. In this frame, our aim is now to establish the gene expression profiles of these different populations by microarrays. RNA amplification and identification will be performed to validate the differential expression of some known genes (i.e. enkephalin , substance P, A_{2A} receptor). Since in the same time, a competing team published similar results Lobo et al. (2006), we will re-oriented our studies by examining the modifications of the specific gene expression profile in this striatopallidal population in pathological conditions as cocaine addiction or dopamine depletion as well as in case of specific gene inactivation (see below).

We have crossed these "striatopallidal-Cre" mice with strains of "floxed" mice allowing the selective inactivation of genes in this population of neurons. The NMDA receptors seem to play a key role in reward and drug addiction (ventral striatum) and in motor learning (dorsal striatum). Moreover, NMDA receptors are supposed to be involved in synaptic plasticity at the cortico-striatal and cortico-accumbal synapses (LTP and LTD). Synaptic plasticity in these areas is strongly suggested to be the basis for motor learning and addiction. An important question is therefore the specific role of this receptor in each subpopulation. The NR1 floxed ($NR1^{f/f}$) mice has been previously generated and published (Tsien et al., 1996) and have been obtained from Prof. Tonegawa and collaborators (MIT, Boston, USA) allowing a conditional inactivation of NR1 by the Cre recombinase. These mice have been double-crossed with our $A_{2A}R$ -Cre mice to obtain homozygous mice. The characterization of $A_{2A}R$-Cre/+ $NR1^{f/f}$ mice by binding autoradiography using a NMDA receptor specific ligand showed a 30% and 40% decrease in binding in the caudate-putamen and accumbens nucleus, respectively, as compared to wild type littermates. On the other hand, there was no differences in NMDA receptor ligand binding between mutant and wild-type in the cerebral cortex suggesting the selectivity of striatal NR1 inactivation. For different technical reasons, in view of the very low number of pups that could be generated, we had to change our breeding strategy in order to obtain a number of animals sufficient for the behavioural analysis.

Another gene of interest in the context of drug addiction and cortico-striatal plasticity is the kinase cdk5. Indeed, the role of this kinase has been previously suggested in the striatal physiology and more specifically in drug addiction (Bibb et al., 2001; Takahashi et al., 2005). However, the cellular target in the striatum remain totally unknown. We have therefore initiated a collaboration Dr Ashok Kulkurani, (NIDCR, NIH Bethesda, USA), who generated floxed cdk5 mice ($cdk5^{f/f}$) (Hirasawa et al., 2004). The $cdk5^{f/f}$ mice that we received, have been backcrossed on a pure C57Bl6 genetic background in order to obtain mice with an homogenous background required for the future experiments, as the behavioral analysis. We have completed the 4^{th} backcross and therefore started breeding with our $A_{2A}R$-Cre/+.

An alternative strategy to examine the specific function of this population of striatopallidal neurons either in drug addiction or motor learning could be to specifically killed them. We took the opportunity of the availability of a mouse strain allowing the inducible expression of the Diphtheria Toxin Receptor (Rosa26-iDTR) (Gropp et al., 2005). In these mice, the expression of human DTR, placed under the control of the ubiquitous promoter ROSA-26, is prevented by a STOP cassette floxed by two LoxP sites. These mice have been crossed with our $A_{2A}R$-Cre. Therefore, in the resulting mice, DTR should specifically be expressed in neurons in which the Cre recombinase is expressed, namely the striatopallidal neurons. When injected to such mice, the Diphteria Toxin (DT) will bind the membrane human DTR. After endocytosis of the complex, it will inhibit protein synthesis leading to cell death. We have tested several protocols for the administration of the toxin (systemic vs. intrastriatal stereotaxic injections) in order to optimalize the resulting neuronal death. We have identify one of these stereotaxic protocols that results in a nearly absence of enkephalin mRNA as detected by in situ hybridization together with the persistence of a normal substance P mRNA levels, suggesting that we succeed in a specific damage of striatopallidal neurons. We will now start the production of these mice and will use this protocol to identify their behavioral defects.

In order to prevent any eventual compensation process that could result from gene inactivation started during development, we have initiated a second and alternative approach aiming to reversibly inactivate these striatopallidal neurons in a specific time window. For this, we used a second transgenic strategy, namely the TRE/tTA expression system which allow to inductively and reversibly control a gene expression in a specific cell population via administration of tetracycline (Kelz et al., 1999). This system is based on two components. On one hand, the gene coding for the transcriptionnal transactivator tTA (tetracycline TransActivator) is placed under the control of a cell-specific promoter. On the other hand, the gene whose expression will inactivate the neurons of interest is placed under the control of the TRE (Tetracycline-Responsive Element) promoter that binds tTA and will be regulated by the presence or absence of tetracycline. We have constructed and generated lines of transgenic mice that should present a targeted expression of the transactivator tTA in striatopallidal neurons. The characterization of these mice is underway. Through the generation of additional mice expressing gene under the control of TRE promoter, we will inactivate the striatopallidal neurons by acting at two different levels. The first aim will be to abolish the neuronal excitability of these neurons by expressing the hyperpolarizing channel, Kir2.1. The second will be to specifically block the intracellular signaling cascade targeting the protein kinase A (PKA) by expressing a specific peptide inhibitor of this kinase, the PKAI. Several founders have been obtained and their characterization is underway.

Regulation of striatal neurons excitability and of corticostriatal transmission and plasticity

We previously studied the modulation of the glutamatergic corticostriatal transmission and plasticity by A_{2A} receptor (D'Alcantara et al., 2001). We now studied the mechanisms of the modulation exerted by D2 and A_{2A} receptors on the striatal neurons excitability and corticostriatal transmission and plasticity in normal and hypodopaminergic conditions. By using the perforated patch configuration of the patch clamp technique in combination with peptide occlusion protocols, we showed that dopamine D2 receptor activation abolished the NMDA-induced down- to up-state transitions and hence striatal neurons excitability. Peptide occlusion showed that this effect occurs through a pathway involving a subtype of calcium channels (CaV1.3). This D2-mediated effect is fully reversed by co-stimulation of A_{2A} receptor although activation of the A_{2A} receptor is unable to modify the down- to up-state transitions (Azdad et al., 2006). This suggests that the action of A_{2A} receptor activation could be completely or partially due to an intramembrane interaction such as D2-A_{2A} heteromerization rather than to activation of an intracellular cascade. Experiments are presently running to test these hypotheses by using occlusion by specific peptides. Preliminary data seem to favor the involvement of D2- A_{2A} heteromerization in this modulation.

Some results have been obtained in A_{2A} knock-out animals and experiments will also be conducted in transgenic rats overexpressing the human A_{2A} receptor that we fully characterized morphologically and behaviorally (Gimenez-Llort et al., 2006).

II. Involvement of the regulation of calcium homeosatsis by calcium binding proteins such as calretinin in the cerebellar physiology

We had characterized cellular mechanisms leading to the alterations observed in calretinin-deficient mice through an approach combining electrophysiology in vitro (patch clamp in the perforated patch configuration) of granular cells of the cerebellum and computer modeling. We demonstrated that the absence of calcium buffering modifies the intrinsic excitability through a modification of the response of calcium-activated potassium channels (Gall et al., 2003). We extended these data by using different mathematical models. Further, we asked whether an increase in neuronal calcium buffering capacity would have consequences on neuronal excitability. Using these mathematical models, we suggested that this increase dramatically changes the firing pattern of modeled granule cell from a regular spiking to different types of bursting firing (Roussel et al., 2006). This theoretical suggestion has been fully verified experimentally by loading different concentrations of the exogenous calcium buffer BAPTA through the patch clamp pipette in granule cells recorded in brain slices (Roussel et al., 2006). This suggests that subtle and local modifications in calcium buffering capacity could dramatically change the mode of neuronal coding .

To more deeply understand the place of these granule cells in the computational network of the cerebellar cortex, members of the laboratory take part to several studies. The first was reported last year and described the molecular mechanisms of long term potentiation induction at the mossy fiber-granule cell synapse (Gall et al., 2005) A second study identified the molecular mechanisms of GABA modulation on granule cell potassium channels. In this work, thanks to the development of multiplex RT-PCR on single cell, we were able to identify inward rectifier potassium channels subtypes expressed in individual granule cells and to correlate these expression profiles with the electrophysiological data (Rossi et al., 2006). Our results suggest that, in granule cells, $GABA_B$ receptors can exert an inhibitory control over constitutive inward rectifier K^+-currents mediated by Kir2 channels.

The absence of calretinin in cerebellar granule cells constitute a main hypothesis consistent with the perturbations that we previously demonstrated in $Cr^{-/-}$ mice (Schiffmann et al., 1999; Gall et al., 2003; Cheron et al., 2005). To investigate this hypothesis, we specifically rescued by cell-specific transgenesis the expression of calretinin in the cerebellar granule cells of $Cr^{-/-}$ mice. The calretinin expression was targeted to cerebellar granule cells by using a fragment of the gene coding for the $GABA_A$ $\alpha 6$ subunit encompassing the promoter and the exons 1 to 8. This part of the gene has been previously shown to allow restricted transgene expression in cerebellar granule cells. We obtained several lines of transgenic $Cr^{-/-}$ mice exhibiting a selective and restricted re-expression of calretinin in granule cells as demonstrated by in situ hybridization, RT-PCR and imunohistochemistry (Bearzatto et al., 2006). In vitro experiments using patch clamp technique in these strains of mice demonstrated that the rescue of calretinin expression in granular cells restores a normal intrinsic excitability of these neurons. Moreover, in vivo electrophysiology experiments demonstrated that the rescue of calretinin in granule cells restores a normal firing behavior of Purkinje cells recorded in alert mice. Finally, behavioral analysis of the motor coordination also showed that the rescue expression of calretinin only in cerebellar granule cells is sufficient to restore a normal phenotype for all parameters (Bearzatto et al., 2006).

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Evaluation of leptin antagonists for treatment of multiple sclerosis

1. Introduction

The cytokine-like hormone leptin is mainly secreted by adipocytes and its level in circulation reflects the body fat status. Leptin acts as an adipostat: leptin levels are sensed and interpreted by the hypothalamic neuron circuitry, leading to an altered energy balance. Spontaneous mutations that lead to a functional defect in the leptin/receptor system thus cause a morbid obese phenotype due to increased food intake and reduced energy expenditure. Besides this body-weight control, leptin also plays an important role in the regulation of immune responses, and possibly in the onset of several auto-immune diseases. Indeed, leptin- or leptin receptor-deficient mice are less affected by or are protected against the development of several inflammatory autoimmune diseases. Examples include multiple sclerosis, rheumatoid arthritis, type I diabetes, and Crohn's disease (Peelman et al. 2004a).

Leptin mediates its effects upon binding and activation of the single-subunit leptin receptor (LR). The LR long form, the only isoform capable of signaling, is highly expressed in hypothalamic neurons, in line with its role in regulating metabolism. In addition, expression at functional levels could also be shown in several other cell types including hepatocytes, immune cells etc., thereby forming the basis for the peripheral biological functions of leptin. Like all members of the class I cytokine receptor family, the LR lacks intrinsic kinase activity and uses cytoplasmic associated Janus kinases (JAKs). Leptin binding leads to JAK activation by inducing their cross-phosphorylation. JAKs then phosphorylate tyrosine residues in the cytosolic tail of the LR allowing coupling to several signaling cascades, including the STAT3, PI3K and ERK pathways (Figure 1). Extracellularly, the LR consists of two so-called cytokine receptor homology (CRH) domains, CRH1 and CRH2. Both are separated by an immunoglobulin-like (Ig-like) domain, and are followed by two additional fibronectin type III (FNIII) modules close to the membrane (Figure 1). CRH2 is the major leptin binding domain (Fong et al. 1998, Zabeau et al. 2004).

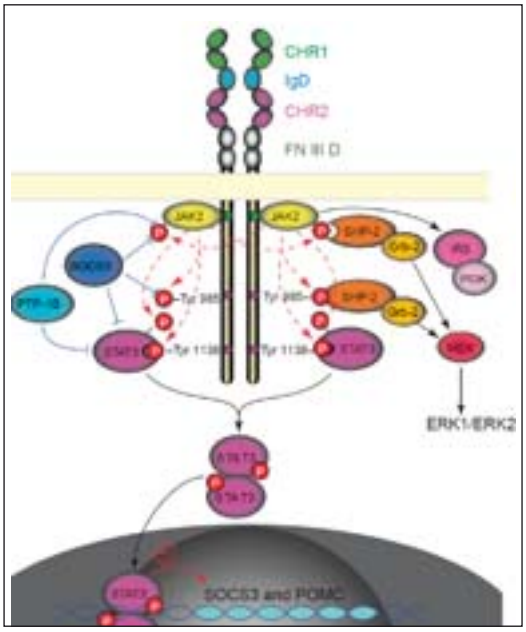

Figure 1: leptin receptor topology and signal transduction mechanism

2. Analysis of leptin receptor complex formation and activation

Understanding LR complex formation and activation is an absolute prerequisite for the design of leptin antagonists. Evidence is accumulating that the LR functions as a tetramer, composed of two cysteine linked dimers. We built a molecular model for the leptin/receptor complex (Figure 2) and proposed a mechanism for activation of the LR. Leptin first binds to a disulfide-linked dimeric LR. Binding site I of leptin interacts with the CRH2 domain of the first LR chain, binding

site II interacts with the CRH2 domain of the second LR chain. Activation of the receptor then requires dimerisation of these 1:2 leptin:LR trimers. This hexamerisation involves interactions of binding site III of the leptin molecule of a first trimer with the LR Ig-like domain of a second trimer (Peelman et al., 2006). In the absence of ligand, the FNIII domains, and therefore also the cytoplasmic receptor tails with the JAKs, are held spatially apart, preventing constitutive, ligand-independent kinase activation. Ligand binding brings the FNIII domains of two different preformed dimers in close proximity, whereby subsequent S-S bridge formation may fix the complex in the correct orientation, leading to signal transduction. Supporting this concept, a deletion variant with an extracellular domain consisting of only the FNIII domains is constitutively active.

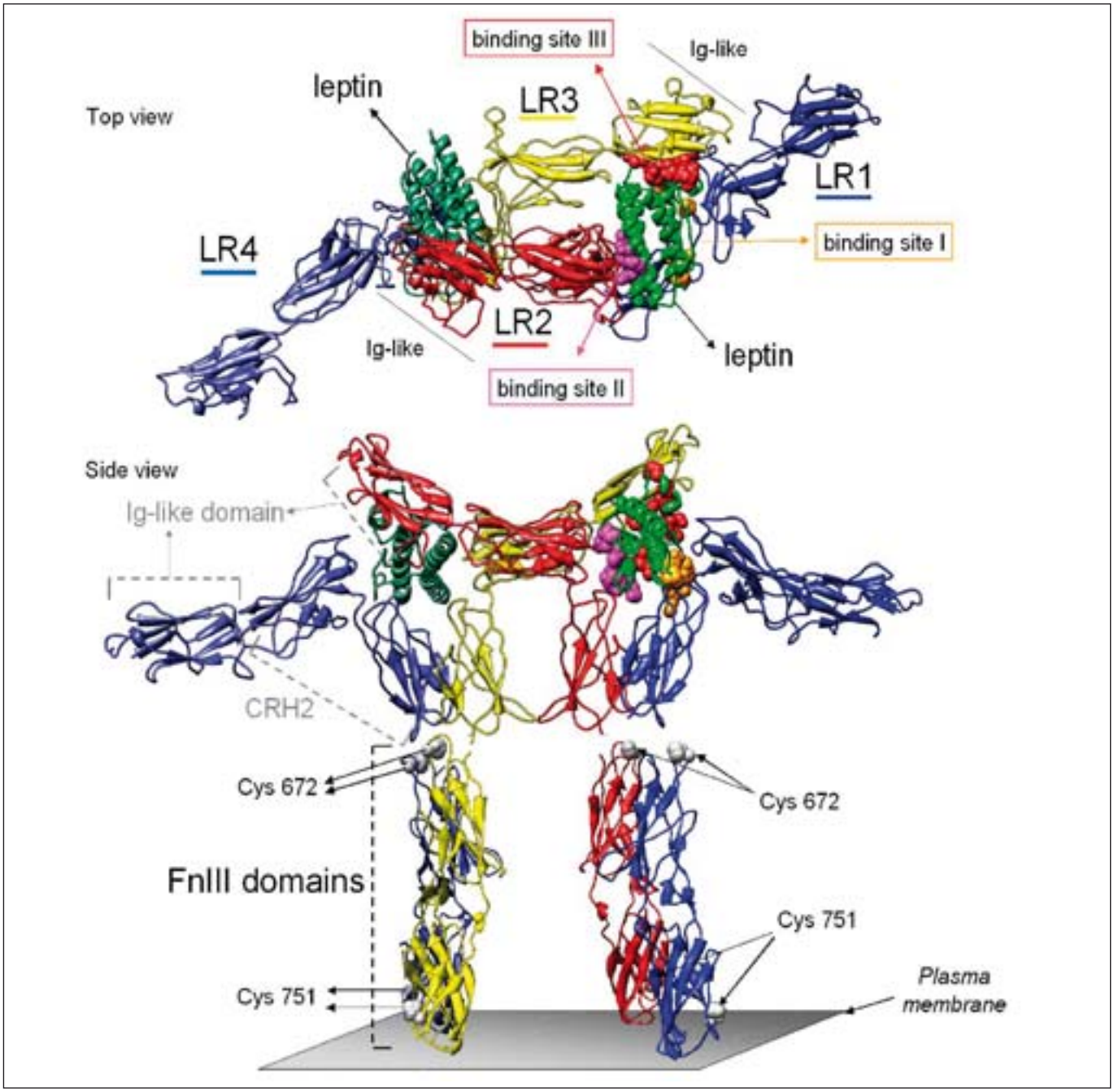

Figure 2: Molecular model for the 2:4 leptin/leptin receptor complex. Each leptin molecule interacts with three different LR chains via three different binding sites. Mutations in binding site II (magenta spheres) affect leptin receptor bindin. Binding site I and III mutations affect LR activation (orange/red spheres)

This model was very recently used to explain the phenotype of four novel leptin receptor mutations in the human population that cause morbid obesity (in collaboration with Dr. Farooqi, Oxford U. UK, manuscript in preparation).

3. Optimisation of leptin muteins with antagonistic properties

Mutations in binding site III of leptin interfere with the hexamerisation process and thus with receptor activation. A S120A/T121A binding site III leptin mutant still binds to the receptor, but is unable to activate the receptor (Peelman et al., 2004b). It therefore acts as a competitive inhibitor of leptin receptor signaling. A purification protocol for the E. coli produced leptin antagonist was established (see previous report).

Given the very short half-life of leptin in circulation (minutes), blocking effects of a leptin antagonist in vivo can only be observed when its half-life is extended (hours). To increase the circulation lifetime of the S120A/T121A leptin antagonist, we tested the possibility of PEGylation. We first attempted to site-specifically add 40 or 60 Kd PEG groups to the S120A/T121A leptin. Two strategies were tested: PEGylation of the N-terminus and of specific cysteine residues, introduced by mutagenesis. The N-terminus was modified using 40 Kd mPEG2-ButyrALD, a butyraldehyde pegylation reagent that preferentially interacts with the N-terminus of proteins. The cysteine residues were pegylated using maleimide (PEG)2, a branched 60 kDa PEG with a maleimide functional group for cysteine modification. Both PEGylation procedures did not yield reproducible results. We therefore switched to PEGylation of lysine residues in leptin, using Y-PEG-NHS 40 K, an N-hydroxy succinimide PEGylation reagent. This procedure is reproducible, with higher yields: Western blot analysis shows a yield of 20-30 % of pegylated leptin (figure 3). Distortion in the right lanes of the gel is caused by the excess PEG.

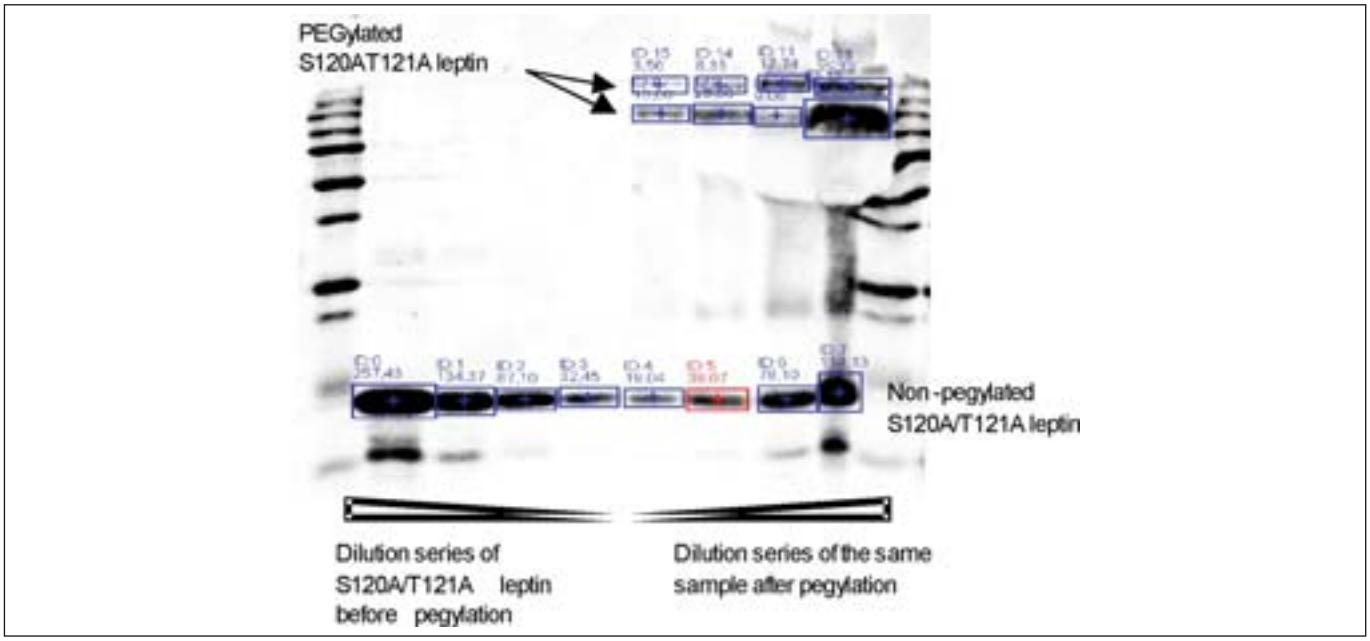

Figure 3: Pegylation of mouse S120A/T121A leptin with Y-PEG-NHS 40K.
Western blot analysis using anti-mouse leptin antibodies and Odyssey Infrared Imager allows quantification of the relative intensity of the bands (numbers above the squares).

Based on this procedure, we will now scale-up production and determine the biological activity of the PEGylated leptin antagonist. If necessary, additional lysine residues will be introduced (e.g. at position 120 or 121, to enhance the antagonistic effect), or will be removed (e.g. at position 15 since PEGylation of this site may interfere with CRH2 binding) to design an optimal antagonist.

4. Development of antagonistic nanobodies targeting the leptin receptor

A concern in the development of leptin-based therapeutic strategies for autoimmune diseases is that complete LR blockage also interferes with leptins hypothalamic body weight regulating role. Indeed, treatment of mice with the S120A/T121A leptin mutein induces significant weight gain (Peelman et al., 2004b). It is likely that the blood-brain-barrier (BBB) can be exploited to separate the peripheral (i.e. on immune cells) and central (i.e. on hypothalamic neurons) activities of leptin. An active leptin BBB transport model has recently been suggested, but the transporter system itself has not yet been identified (Banks et al., 2002). Possibly, a short isoform of the LR that lacks most of the cytosolic domain plays a modulating role by trapping and transferring the ligand to the actual transporter system. We aim to develop a leptin antagonist that does not interfere with leptin transport, and that cannot cross the BBB itself. Such antagonist may selectively inhibit the peripheral functions of leptin. Based on our hexameric leptin/LR model, we postulated that molecules directed against the LR FNIII or Ig-like domains are good candidates to fulfill such role.

We chose to use the "nanobody" technology for the development of such LR antagonists. Nanobodies are the variable domain of a class of antibodies found in camels and llamas that are only composed of heavy chains. Nanobodies have relatively long complementarity determining regions (CDRs) that allow binding to regions that lie deeper inside protein complexes (Desmyter et al. 1996). Llamas were immunized with affinity purified extracellular part of the murine LR (in collaboration with Ablynx). Blood was collected, lymphocytes isolated, and nanobody sequences were cloned in an expression-vector that allows bacteriophage-surface expression. This strategy allows selection of high-affinity binders. Nanobodies were initially tested for neutralization of leptin signaling in LR-transfected Hek293T cells using the STAT3-responsive rPAP1-luciferase reporter. Neutralizing nanobodies were then further evaluated in a leptin-binding assay. Therefore, cells were transfected with the receptor and incubated with SEAP-labeled leptin (SEAP: secreted alkaline phosphatase, offers an easy enzymatic read-out for leptin binding) in the presence or absence of the nanobodies. These experiments showed that not all neutralizing nanobodies interfered with ligand binding. We next used the different LR sub-domains fused to SEAP to determine the specificity of the selected nanobodies. We found that all neutralizing nanobodies were directed against either the CRH2, Ig-like or FNIII domains, exactly as was predicted by our model.

Three nanobodies, cam 2.17 (directed against CRH2), cam 4.10 (against Ig-like) and cam 4.11 (against FNIII), were selected for initial *in vivo* analysis. Therefore, nanobody - murine albumin constructs were generated, expressed in Cos-1 cells and purified using the N-terminal HA-tag. Such fusion proteins remain longer in circulation, and accumulate at sites of inflammation. The nanobody-albumin chimeras retained their leptin neutralizing properties. Fusion-proteins were

injected in 9 week old C57Bl/6 mice according to the following scheme: day 0-3: a single injection of 20 µg/mouse/day; day 4 – 13: single injection of 10 µg/mouse/day. Murine albumin (same injection-scheme) and PBS served as negative controls. The S120A/T121A leptin antagonist (see above) was used as positive control. To avoid rapid clearance of this mutant, the non-neutralizing 2A5 antibody was co-injected. Mice were weighed daily (figure 4, panel A), and at day 14 treated with α-CD3 (10 µg per mouse). Serum levels of T_H1 (INF-γ) and T_H2 (IL-4) cytokines were determined in a series of ELISA experiments (panels B and C).

None of the evaluated nanobody-albumin fusion proteins had a significant effect on body weight. Serum cytokine levels (IL4 and IFNγ) are elevated upon αCD3 challenge in animals that received the nanobodies, suggesting their *in vivo* efficacy. Additional experiments are required to corroborate these findings. It is of note that cam 2.17 is directed against the CRH2 domain, and interferes with leptin binding *in vitro*. This observation might suggest that the role of the LR short form in the active transport of leptin across the BBB (see above) is restricted or is merely modulating by nature. This was previously suggested by Banks and co-workers, based on a set of experiments with Koletsky rats (Banks *et al*., 2002). Due to a nonsense mutation, these animals do not express membrane-bound LRs. The weight gain of S120A/T121A treated mice implies that the mutant works centrally, and thus is actively transported over the BBB.

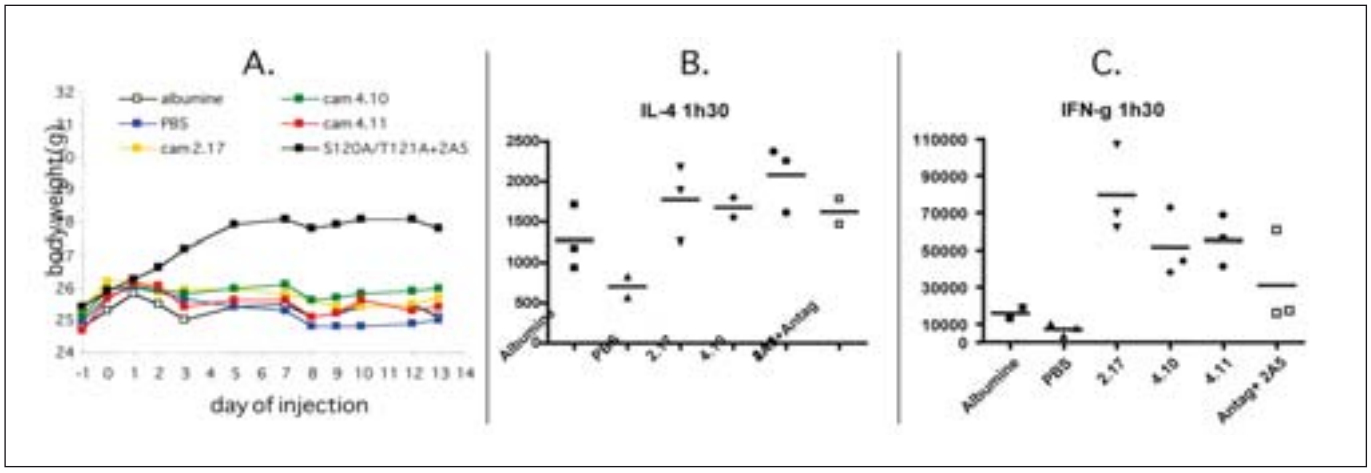

Figure 4: *in vivo* evaluation of three different nanobodies and the S120S/T121A leptin mutant

Although straightforward, the use of nanobodies produced in eukaryotic cells is costly and labour-intensive. We therefore generated double nanobodies that can easily be produced in bacterial expression-systems. The three selected nanobodies (cam 2.17, 4.10, and 4.11) were cloned in a Pax51 vector in tandem with an anti-murine albumin nanobody, a C-terminal 6x His-tag, and a periplasmatic secretion sequence. Fusion proteins were isolated from periplasmatic extracts, and further purified using a nickel metal affinity resin. Average yield was about 1 to 3 mg protein from 1 liter culture. Purified double nanobodies were tested in a sandwich assay in microriterplates coated with murine albumin, with detection using the soluble extracellular domain of the LR (or the β chain in the IL-3 receptor, as a negative control) fused to SEAP. Results in figure 5 illustrate that such nanobodies bind both albumin and the LR. Their neutralizing capacity will be tested in the near future on Ba/F-3 cells stably expressing the LR.

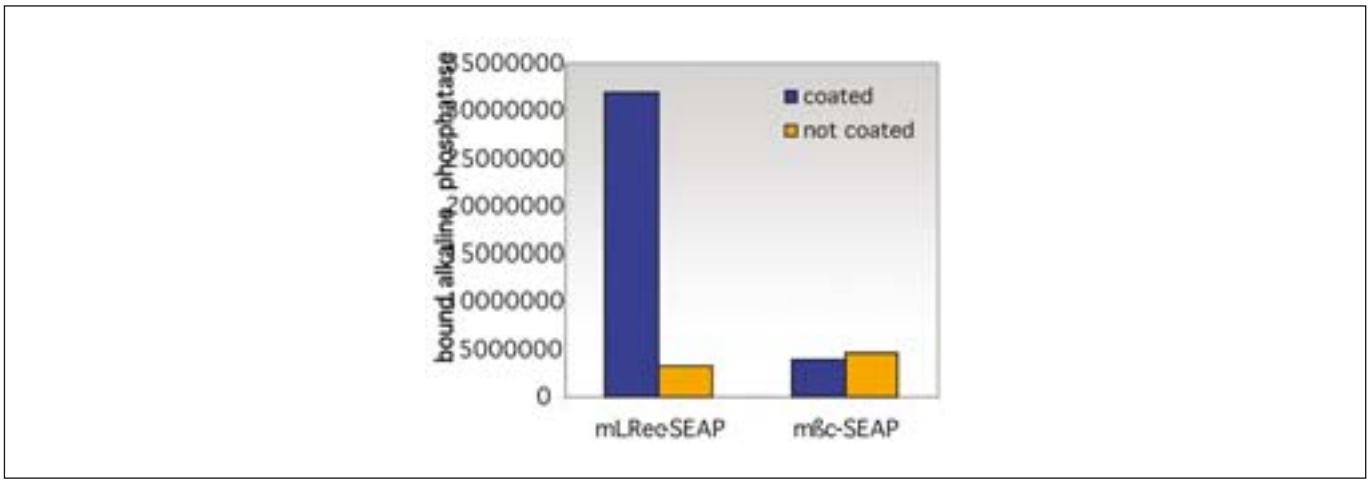

Figure 5: binding and properties of the double nanobodies

5. Future prospects

First, the different antagonists will be evaluated in more detail *in vivo*. The effect on body weight regulation and on immune function in normal mice will first be monitored to further corroborate their mode of action, e.g. selective peripheral vs. central activity. Based on these observations, large-scale production of selected antagonists will be initiated. Later on, these proteins will be analyzed in model systems for multiple sclerosis.

Since we have obtained nanobodies directed against every relevant sub-domain of the LR, these can also be useful tools to study the transport of leptin across the BBB. A relatively straightforward transcytosis assay in MDCK will first be used to set up the conditions for leptin transcytosis. MDCK cells were therefore already stably transfected with the short isoform of the LR. At a later stage, we will extend these studies to a physiologically more relevant assay for leptin BBB transport using rat brain micro-capillaries.

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7. Publications directly related to the project

- PEELMAN, F.*, ISERENTANT, H.*, EYCKERMAN, S., ZABEAU, L. & TAVERNIER, J.
  Leptin, immune responses and autoimmune disease: perspectives on the use of leptin antagonists.
  * Shared co-authorship
  Curr. Pharm. Des. 11, 539-548 (2005) (IF=5.6).
- ISERENTANT, H.*, PEELMAN, F.*, DEFEAU, D., VANDEKERCKHOVE, J.,
  ZABEAU, L. & TAVERNIER, J.
  * shared co-authorship
  Mapping of the interface between leptin and the leptin receptor CRH2 domain.
  J. Cell Sci. 118, 2519-2527 (2005) (IF=7.3).
- ZABEAU, L., DEFEAU, D., ISERENTANT, H., VANDEKERCKHOVE, J., PEELMAN, F. & TAVERNIER, J.
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  New pharmacological perspectives for the leptin receptor.
  Trends Pharmacol Sci, 27, 218-225 (2006) (IF=10.4).
- PEELMAN, F.*, ISERENTANT, H.*, DESMET, A.-S., VANDEKERCKHOVE, J., ZABEAU, L. & TAVERNIER, J.
  * shared co-authorship
  Mapping of Binding Site III in the leptin receptor and modeling of a hexameric leptin: receptor complex.
  J. Biol. Chem., 281, 15496-15504 (2006) (IF=5.9).

Progress Report of the Research Group of

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Molecular genetics and biology of Charcot-Marie-Tooth neuropathies

Introduction

In 2006 we were co-editors of a special review issue of NeuroMolecular Medicine which covers in 17 review articles the current knowledge on the clinical, electrophysiological and pathological aspects of Charcot-Marie-Tooth (CMT) and related disorders. The reviews also synthesize the most recent findings in molecular genetics, biology and therapy for inherited peripheral neuropathies. The pathomechanism of mutant proteins is discussed and correlations are made between the different neuropathy phenotypes and model organisms. Sections are dedicated to the interaction between Schwann cells and the axon in the developing nervous system, and to the role of the immune system related to the pathomechanism. Finally, future perspectives are presented regarding diagnostic and therapeutic approaches based on the molecular findings. The issue can be downloaded from the International Charcot-Marie-Tooth Association (CMTA) website (accessible through the links Researchers/Clinicians: http://www.charcot-marie-tooth.org/Physicians/Physicians_Main.php).

Identification of a novel gene for intermediate CMT neuropathy: YARS

Dominant intermediate CMT (DI-CMT) is a genetic and phenotypic variant of classical CMT characterized by intermediate nerve conduction velocities (NCV) and histological evidence of both axonal and demyelinating features. It is a clinically and genetically heterogeneous entity. In some patients specific mutations in the MPZ or NEFL genes were found. In addition, female and male patients with mutations in the GJB1 gene can display a wide range of nerve conduction velocities. The first evidence that intermediate CMT represents a separate genetic entity was published in 2001, when our group and Australian colleagues independently reported linkage results for two large pedigrees with affected members showing intermediate NCVs. We mapped the DI-CMTA locus to chromosome 10q24.1-q25.1 in a large Italian family. The DI-CMTB locus was mapped to 19p12-p13.2 in an Australian pedigree. We subsequently contributed to the identification of DNM2 as the disease-causing gene in DI-CMTB. DNM2 belongs to the family of large GTPases and is part of the cellular fusion-fission apparatus. Interestingly, in the Australian and Belgian families, which carry two different mutations affecting the same amino acid (Lys558), CMT co-segregated with neutropenia, which had not previously been associated with CMT.

We assigned the DI-CMTC locus to 1p34-p35 in two large pedigrees from Bulgaria and North America. In 2006, we identified three different disease-associated mutations in YARS in DI-CMTC patients from Bulgaria, North America and Belgium. Tyrosyl-tRNA-synthetase (YARS) is essential for protein synthesis, catalyzing the aminoacylation of $tRNA^{TYR}$ with tyrosine. It is the second tRNA-synthetase associated with inherited peripheral neuropathy. Dominant mutations in GARS, encoding glycyl-tRNA synthetase, were found in CMT2D and distal hereditary motor neuropathy type V, two allelic neuropathies assigned to chromosome 7p14. These findings support that aminoacyl-tRNA-synthetases (AARS) and other protein synthesizing complexes may play a role in neurodegeneration.

It was recently demonstrated that under apoptotic conditions the human full-length YARS is secreted and has cytokine activities in addition to its role in protein synthesis. The holoenzyme is a homodimer, with each monomer composed of two fragments having different biochemical properties. The N-terminus (mini-YARS) is the conserved catalytic part of the protein involved in aminoacylation. In addition mini-YARS has an interleukin-8-like activity, stimulates migration of polymorphonuclear cells, and acts as a pro-angiogenic factor. The C-terminal part of YARS is specific for higher eukaryotes, shares high sequence similarity with endothelial monocyte-activating polypeptide II (EMAP II), and also has cell signaling functions.

We performed functional studies of two missense mutations (G41R and E196K) that lie in the catalytic domain of the YARS protein. Our biochemical experiments and genetic complementation in yeast demonstrated partial loss of aminoacylation activity of the mutant proteins. Moreover, mutant YARS, or its yeast orthologue TYS1, have a dominant-negative effect on the function of the wild type protein. Co-immunoprecipitation experiments demonstrated heterodimer formation between the wild type and mutant YARS isoforms that could lower the holoenzyme protein activity, leading to the reduced yeast growth we observed.

We found differences in YARS distribution between neuronal and non-neuronal cells, and between undifferentiated and differentiating neuronal cells. Interestingly, YARS concentrated in granular structures in the growth cone, branch points and the most distal part of projecting neurites in differentiating N2a cells. Anti-YARS staining in primary embryonic (E14) motor neurons also showed that YARS was localized not only throughout the cell body but also in the smallest filopodia projecting from the main neurite. This particular YARS distribution was not observed for arginyl-tRNA- and tryptophanyl-tRNA- synthetases (Jordanova et al. Nature Genetics 2006;38:197-200).

Hereditary sensory neuropathies

We reviewed the genetic advances of hereditary sensory neuropathies (HSN) and hereditary sensory and autonomic neuropathies (HSAN), and summarized all available information on phenotype-genotype correlation and on possible underlying pathomechanisms. These disorders are characterized by prominent sensory loss with acro-mutilating complications and a variable degree of motor and autonomic disturbances. Based on age at onset, clinical features and mode of inheritance, these disorders have originally been subdivided into five types. The identification of eight loci and six disease-causing genes for this group of disorders, however, has shown that this present classification has to be refined. We discussed each of the different loci and genes of these disorders, showing glimpses into a possible underlying pathomechanism leading to the degeneration of sensory and autonomic neurons (Verhoeven et al. Current Opinion in Neurobiology, 2006; 19(5):474-480).

Hereditary sensory and autonomic neuropathy type II (HSAN-II) is caused by recessive mutations in the HSN2 gene assigned to chromosome 12p13.33. We reported three unrelated HSAN-II families with homozygous or compound heterozygous mutations resulting in the truncation of the HSN2 protein. Genotype-phenotype correlations indicated that HSN2 mutations are associated with an

early childhood onset of a predominantly sensory neuropathy, complicated by acromutilations in both upper and lower limbs (Coen et al. Neurology 2006;66:748-751). Congenital insensitivity to pain with anhidrosis (CIPA) or hereditary sensory and autonomic neuropathy type IV (HSAN IV) is the first human genetic disorder implicated in the neurotrophin signal transduction pathway. HSAN IV is characterized by absence of reaction to noxious stimuli, recurrent episodes of fever, anhidrosis, self-mutilating behavior and often mental retardation. Mutations in the neurotrophic tyrosine kinase, receptor, type 1 (NTRK1) are associated with this disorder. We reported four homozygous mutations, two frameshift (p.Gln626fsX6 and p.Gly181fsX58), one missense (p.Arg761Trp) and one splice site (c.359+5G>T) mutation in four HSAN IV patients. The splice site mutation caused skipping of exons 2 and 3 in patient's mRNA resulting in an in-frame deletion of the second leucine-rich motif. NTRK1 mutations are only rarely reported in the European population. This finding extended the spectrum of NTRK1 mutations observed in patients diagnosed with HSAN IV (Verpoorten et al. Neuromuscular Disorders 2006;16:19-25).

Hereditary motor neuropathies

The number of genes associated with motor neuron degeneration has increased considerably over the past few years. As more gene mutations are identified, the hope arise that certain common themes and/or pathways become clear. In a review we focused on recent discoveries related to amyotrophic lateral sclerosis (ALS), spinal muscular atrophies (SMA), and distal hereditary motor neuropathies (distal HMN). It is striking that many of the mutated genes that were linked to these diseases encode proteins that are either directly or indirectly involved in axonal transport or play a role in RNA metabolism. We hypothesized that both phenomena are not only crucial for the normal functioning of motor neurons, but that they could also be interconnected. In analogy with the situation after acute stress, axonal mRNA translation followed by retrograde transport of the signal back to the nucleus could play an important role in chronic motor neuron diseases. Information on the genetic causes of these diseases and the insight into the pathologic processes involved, could ultimately lead to therapeutic strategies that prevent or at least slow this degenerative process (Van Den Bosch and Timmerman, Current Neurology and Neuroscience Reports 2006;6:423-431).

The HMN are a heterogeneous group of disorders characterized by an exclusive involvement of the motor part of the peripheral nervous system. They are usually subdivided in proximal HMN, i.e., the classical spinal muscular atrophy syndromes and distal HMN that clinically resemble CMT syndromes. The distal HMN are clinically and genetically heterogeneous and were initially subdivided in seven subtypes according to mode of inheritance, age at onset, and clinical evolution. Recent studies have shown that these subtypes are still heterogeneous at the molecular genetic level and novel clinical and genetic entities have been delineated. Since the introduction of positional cloning, 13 chromosomal loci and seven disease-associated genes have been identified for autosomal-dominant, autosomal-recessive, and X-linked recessive distal HMN. Most of the genes involved encode protein with housekeeping functions, such as RNA processing, translation synthesis, stress response, apoptosis, and others code for proteins involved in retrograde survival. Motor neurons of the anterior horn of the spinal cord seems to be vulnerable to defects in

these housekeeping proteins, likely because their large axons have higher metabolic requirements for maintenance, transport over long distances and precise connectivity. Understanding the molecular pathomechanisms for mutations in these genes that are ubiquitous expressed will help unravel the neuronal mechanisms that underlie motor neuropathies leading to denervation of distal limb muscles, and might generate new insights for future therapeutic strategies (Irobi et al. Neuromolecular Medicine 2006;8:131-146).

Charcot-Marie-Tooth type 2

Mutations in mitofusin 2 (MFN2) have been reported in Charcot-Marie-Tooth type 2 (CMT2) families. To study the distribution of mutations in MFN2 we have screened 323 families and isolated patients with distinct CMT phenotypes. In 29 probands, we identified 22 distinct MFN2 mutations, and 14 of these mutations have not been reported before. All mutations were located in the cytoplasmic domains of the MFN2 protein. Patients presented with a classical but rather severe CMT phenotype, since 28% of them were wheelchair-dependent. Some had additional features as optic atrophy. Most patients had an early onset and severe disease status, whereas a smaller group experienced a later onset and milder disease course. Electrophysiological data showed in the majority of patients normal to slightly reduced NCVs with often severely reduced amplitudes of the compound motor and sensory nerve action potentials. Examination of sural nerve specimens showed loss of large myelinated fibres and degenerative mitochondrial changes. In patients with a documented family history of CMT2 the frequency of MFN2 mutations was 33% indicating that MFN2 mutations are a major cause in this population (Verhoeven et al. Brain 2006;129:2093-2102).

CMT neuropathy with visual impairment due to optic atrophy has been designated as hereditary motor and sensory neuropathy type VI (HMSN VI). Reports of affected families have indicated autosomal dominant and recessive forms, but the genetic cause of this disease has remained elusive. We studied six HMSN VI families with a subacute onset of optic atrophy and subsequent slow recovery of visual acuity in 60% of the patients. Detailed clinical and genetic studies were performed. In each pedigree, we identified a unique mutation in the gene mitofusin 2 (MFN2). In three families, the MFN2 mutation occurred *de novo*; in two families the mutation was subsequently transmitted from father to son indicating autosomal dominant inheritance. MFN2 is a mitochondrial membrane protein that was recently reported to cause axonal CMT type 2A. It is intriguing that MFN2 shows functional overlap with optic atrophy 1 (OPA1), the protein underlying the most common form of autosomal dominant optic atrophy, and mitochondrial encoded oxidative phosphorylation components as seen in Leber's hereditary optic atrophy. We concluded that autosomal dominant HMSN VI is caused by mutations in MFN2, emphasizing the important role of mitochondrial function for both optic atrophies and peripheral neuropathies (Züchner et al., Annals of Neurology 2006;59:276-281).

Research Articles in 2006 with acknowledgement to the GSKE (see publications enclosed):

- Coen,K., Pareyson,D., Auer-Grumbach,M., Buyse,G., Goemans,N., Claeys,K., Laurà,M., Salmhofer,W., Pieber,T., Nelis,E., De Jonghe,P., Timmerman,V.: Further evidence for mutations in the HSN2 gene causing hereditary sensory and autonomic neuropathy type II. Neurology 66: 748-751 (2006) (I.F.: 5.065)
- Irobi,J., Dierick,I., Jordanova,A., Claeys,K.G., De Jonghe,P., Timmerman,V.: Unraveling the genetics of distal hereditary motor neuronopathies. Neuromolecular Medicine 8(1-2): 131-146 (2006) (I.F.: 4.07)
- Jordanova,A., Irobi,J., Thomas,F.P., Van Dijck,P., Meerschaert,K., Dewil,M., Jacobs,A., De Vriendt,E., Dierick,I., Van Gerwen,V., Guerguelcheva,V., Rao,C.V., Tournev,I., Gondim,F.A.A., D'Hooghe,M., Callaerts,P., Van Den Bosch,L., Timmermans,J-P., Robberecht,W., Gettemans,J., Thevelein,J., De Jonghe,P., Kremensky,I., Timmerman,V.: Disrupted function and axonal distribution of mutant tyrosyl-tRNA synthetase associated with dominant intermediate Charcot-Marie-Tooth neuropathy. Nature Genetics 38(2): 197-202 (2006) (I.F.: 25.797)
- Van Den Bosch,L., Timmerman,V.: Genetics of motor neuron disease. Current Neurology and Neuroscience Reports 6: 423-431 (2006)
- Verhoeven,K., Claeys,K.G., Züchner,S., Schröder,J.M., Weis,J., Ceuterick,C., Jordanova,A., Nelis,E., De Vriendt,E., Van Hul,M., Seeman,P., Mazanec,R., Mustafa,S.G., Szigeti,K., Mancias,P., Butler,I., Kochañski,A., De Bleecker,J., Van den Bergh,P., Verellen,C., Van Coster,R., Rasic,V., Nevo,Y., Roelens,F., Vieregge,P., Vinci,P., Moreno,M.T., Christen,H-J., Shy,M.E., Lupski,J.R., Vance,J.M., De Jonghe,P., Timmerman,V.: MFN2 mutation distribution and genotype/phenotype correlation in Charcot-Marie-Tooth type 2. Brain 129(Pt 8): 2093-2102 (2006) (I.F.: 7.535)
- Verhoeven,K., Timmerman,V., Mauko,B., Pieber,T., De Jonghe,P., Auer-Grumbach,M.: Recent advances in hereditary sensory and autonomic neuropathies. Current Opinion in Neurology 19(5): 474-480 (2006) (I.F.: 4.873)
- Verpoorten,N., Claeys,K.G., Deprez,L., Jacobs,A., Van Gerwen,V., Lagae,L., Arts,W.F., De Meirleir,L., Ceuterick,C., De Jonghe,P., Timmerman,V., Nelis,E.: Novel frameshift and splice site mutations in the neurotrophic tyrosine kinase receptor type 1 gene (NTRK1) associated with hereditary sensory neuropathy type IV. Neuromuscular Disorders 16(1): 19-25 (2006) (I.F.: 3.34)
- Züchner,S., De Jonghe,P., Jordanova,A., Claeys,K.G., Guerguelcheva,V., Cherninkova,S., Hamilton,S. R., Staijch,J., Tournev,I., Verhoeven,K., Pericak-Vance,M., Bird,T., Timmerman,V., Shy,M., Vance,J.M.: Axonal neuropathy with optic atrophy is caused by mutations in mitofusin 2. Annals of Neurology 59(2): 276-281 (2006) (I.F.: 7.571)

Other publications in 2006:

- Lupski, J.R. and Timmerman, V.: The CMT1A duplication – an historical perspective viewed from two sides of an ocean. In "Genomic Disorders – The Genomic Basis of Disease" (J.R. Lupski and P. Stankiewicz, Eds), Humana Press, Totowa, N.J., U.S.A. 2006
- Reilly,M., De Jonghe,P., Pareyson,D.: 136th ENMC International Workshop: Charcot-Marie-Tooth disease type 1A (CMT1A); 8-10 April 2005, Naarden, The Netherlands. Neuromuscular Disorders 6(6): 396-402. (2006) (I.F.: 3.34)
- Timmerman, V. and Lupski, J.R. The CMT1A duplication and HNPP deletion. In "Genomic Disorders – The Genomic Basis of Disease" (J.R. Lupski and P. Stankiewicz, Eds) Humana Press, Totowa, N.J., U.S.A. 2006
- Timmerman,V., Hermann,D.N.: A 'nerve' ending story in the identification of mutations in Charcot-Marie-Tooth neuropathy. Neurology 67(7): 1114-1115 (I.F.: 5.065)
- Timmerman,V., Lupski,J., De Jonghe,P.: Molecular genetics, biology, and therapy for inherited peripheral neuropathies. Neuromolecular Medicine 8(1-2): 1-2 (2006) (I.F.: 4.07)
- Verpoorten,N., De Jonghe,P., Timmerman,V.: Disease mechanisms in hereditary sensory and autonomic neuropathies. Neurobiology of Disease 21(2): 247-255 (2006) (I.F.: 4.048)

Research Activities 2006:

Scientific Prizes:

- **De Jonghe P.:** Three-yearly award (2006) Prof. Dr. Raymond Van den Bergh for Neurology, Neurosurgery en Psychiatry. Vereniging van Vlaamse Zenuwartsen (VVZ). "Clinical, electrophysiological and molecular genetic aspects of inherited peripheral neuropathies".
- **Timmerman V.:** Prijs van de Stiching Antoine Faes voor onderzoek in Biomedische Wetenschappen, Turnhout, 5 oktober 2006: "Inherited peripheral neuropathies: identification of loci and genes through molecular genetic and biological research".

Awards and fellowships:

- **Claeys K.:** Fellowship of the World Muscle Society, Bruges October 4-7, 2006
- **Dierick I., Irobi J. and Verhoeven K.:** Travel Awards of the Fund for Scientific Research (FWO-Vlaanderen) to attend the Conference on Molecular and Cellular Mechanisms of Axon Degeneration, The Babraham Institute, Cambridge, UK September 11-12, 2006

PhD theses:

- **Nathalie Verpoorten:** Identification of novel genes for primary sensory neurons and Molecular genetics of sensory and motor neuropathies. PhD in Biomedical Sciences. Public defence: 25/01/2006, supervisors: V. Timmerman and P. De Jonghe
- **Gregor Kuhlenbäumer:** Identification of the genetic defect causing hereditary neuralgic amyotrophy (HNA). PhD in Biomedical Sciences. Public defence: 27/10/2006, supervisors: C. Van Broeckhoven and V. Timmerman

Master theses:

- **Ellen Claesen:** Mutatie- en koppelingsanalyse in families met erfelijke motorische neuropathieën. Academic Year 2005-2006. Master in Biology. Supervisors: V. Timmerman and J. Irobi
- **Stefaan Felix:** Mutatieanalyse van het DCTN1-gen in patiënten met een distale hereditaire motorische neuropathie. Academic Year 2005-2006. Graduation report.
- Supervisors: V. Timmerman and E. De Vriendt

Presentations:

Chair and Organizational Activities:

International

- **De Jonghe P.**: Member of the local organizing committee: 11th International Congress of the World Muscle Society. Brugge, Belgium, October 4-7, 2006
- **De Jonghe P.**: the XI. International Congress on Neuromuscular Diseases: Chair-Symposium Peripheral Neuropathies. Istanbul, Turkey, 2-7 July, 2006
- **Timmerman V.**: Member of the Organising and Scientific Committee: 11th European Drosophila Neurobiology Conference, Leuven, Belgium, September 2-6, 2006
- **Timmerman V.**: Chair: Posters 7 Neuropathies II, 11th International Congress of the World Muscle Society, Brugge, Belgium, October 4-7, 2006
- **Timmerman V.:** member of discussion forum for the NIH Peripheral Neuropathy Workshop, October 22-24, 2006, Bethesda, MD, USA

National

- **Timmerman V.**: Chair: 'Science Club: Neuroscience', VIB Seminar 2006, Blankenberge, Belgium, March 9-10, 2006

Invited Lectures:

International

- **De Jonghe P.**: the XI. International Congress on Neuromuscular Diseases. "Hereditary motor neuropathies " Istanbul, Turkey, 2-7 July, 2006
- **De Jonghe P.**: the XI. International Congress on Neuromuscular Diseases. "New horizons in hereditary neuropathies" Teaching course. Istanbul, Turkey, 2-7 July, 2006
- **De Jonghe P.**: 11th International Congress of the World Muscle Society: 'The never ending story of genetics of inherited neuropathies', Brugge, Belgium, October 4-7, 2006
- **Irobi J** : About nerves, pedigree and genes: Molecular Genetics of Inherited Peripheral Neuropathies. Faculty of Medicine Seminar, University of Ibadan, Nigeria, August 16, 2006
- **Jordanova A.**: Aminoacyl-tRNA-synthetases in health and disease, Disrupted function and axonal distribution of mutant tyrosyl-tRNA synthetase in dominant intermediate Charcot-Marie-Tooth neuropathy. Seminar, The Scripps Research Institute, San Diego, CA USA, June 2-7, 2006
- **Jordanova A.**: Involvement of AARSs in disease and therapeutics, Disrupted function and axonal distribution of mutant tyrosyl-tRNA synthetase in dominant intermediate Charcot-Marie-Tooth neuropathy. 2006 International Conference on Aminoacyl-tRNA-synthetases: From the Genetic Code to Human Disease & Medicine, San Diego, CA USA, October 1-6, 2006
- **Nelis E.**: Molecular genetic diagnostics of polyneuropathies. Neuromuscular Meeting – Polyneuropathies, Helsinki, Finland, November 17, 2006

- **Timmerman V.**: Symposium on Neuromuscular Disorders: VI Simposio internazionale di genetica clinica e molecolare – Basi genetico-molecolari di malattie neuromuscolari: 'About nerves, pedigrees and genes: molecular genetics of inherited peripheral neuropathies', Rome, Italy, May 19, 2006
- **Timmerman V.**: Aminoacyl-tRNA-synthetases in health and disease, About nerves, pedigrees and genes: molecular genetics of inherited peripheral neuropathies. Seminar, The Scripps Research Institute, San Diego, CA USA, June 2-7, 2006

National

- **Irobi J.:** Functional characterization of HSP22 mutations associated with axonal degeneration, VIB Science Club Neurodegenerative Diseases, Leuven, Belgium, October 6, 2006
- **Timmerman V.:** Molecular genetics and biology of inherited peripheral neuropathies: a "nerve" ending story? Centrum Medische Genetica, Universiteit Gent, 28 November 2006

Oral Presentations – Slide Sessions:

International

- **Claeys K.**: 11th International Congress of the World Muscle Society: 'Dynamin 2 mutations are associated with dominant intermediate Charcot-Marie-Tooth disease and dominant centronuclear myopathy', Brugge, Belgium, October 4-7, 2006
- **Irobi J**.: In vitro expression of small heat shock protein HSPB8 and HSPB1 mutations causing axonal neuropathy. 6th Dutch Chaperone meeting, UMCG Groningen, The Netherlands, February 24, 2006
- **Irobi J**.: In vitro expression of small heat shock protein HSPB8 and HSPB1 mutations causing axonal neuropathy, 11th International Congress of the World Muscle Society, Brugge, Belgium, October 4-7, 2006
- **Jordanova A**.: Disrupted function and axonal distribution of mutant tyrosyl-tRNA synthetase in dominant intermediate Charcot-Marie-Tooth neuropathy. European Conference of Human Genetics 2006, Amsterdam, The Netherlands, May 6-9, 2006

National

- **Jordanova A**.: Disrupted function and axonal distribution of mutant tyrosyl-tRNA synthetase in dominant intermediate Charcot-Marie-Tooth neuropathy. VIB Seminar 2006, Blankenberge, Belgium, March 9-10, 2006

Poster Presentations:

International

- **Dierick I.**: In vitro aggregation of stress proteins associated with inherited distal motor neuropathies. 6th Dutch Chaperone meeting, UMCG Groningen, The Netherlands, February 24, 2006
- **Dierick I.**: In vitro aggregation of stress proteins is associated with inherited motor neuropathies. Molecular and Cellular Mechanisms of Axon Degeneration, Cambridge, UK, September 10-12: 18, 2006
- **Gonçalves R.:** Tyrosyl-tRNA synthetase (YARS), molecular genetics and functional studies on a dominant intermediate Charcot-Marie-Tooth associated gene. Molecular and Cellular Mechanisms of Axon Degeneration, Cambridge, UK, September 10-12: 20, 2006
- **Irobi J**.: In vitro expression af small heat shcok protein HSPB8 and HSPB1 mutations causing axonal neuropathy. Molecular and Cellular Mechanisms of Axon Degeneration, Cambridge, UK, September 10-12: 17, 2006

- **Verhoeven K**.: A major cause for autosomal dominant axonal CMT neuropathy: MFN2 mutations. Molecular and Cellular Mechanisms of Axon Degeneration, Cambridge, UK, September 10-12: 19, 2006
- **Verhoeven K**.: 11th International Congress of the World Muscle Society: 'Mitofusin 2 mutations are a major cause for autosomal dominant axonal CMT neuropathy', Brugge, Belgium, October 4-7, 2006

National

- **Dierick I.**: In vitro aggregation of stress proteins associated with inherited distal motor neuropathies. VIB Seminar 2006, Blankenberge, Belgium, March 9-10, 2006
- **Dierick I.**: In vitro aggregation of stress proteins associated with inherited distal motor neuropathies. Spring meeting of the Belgian Society for Cell and Developmental Biology: Molecular and cellular basis of neuroconnectivity, Leuven, Belgium, May 4-6, 2006
- **Gonçalves R.:** Tyrosyl-TRNA synthetase (YARS), molecular genetic and functional studies of a dominant intermediate Charcot-Marie-Tooth associated gene. VIB Seminar 2006, Blankenberge, Belgium, March 9-10, 2006
- **Irobi J**.: In vitro expression of small heat shock protein HSPB8 and HSPB1 mutations causing axonal neuropathy. VIB Seminar 2006, Blankenberge, Belgium, March 9-10, 2006
- **Irobi J**.: In vitro expression of small heat shock protein HSPB8 and HSPB1 mutations causing axonal neuropathy. Spring meeting of the Belgian Society for Cell and Developmental Biology: Molecular and cellular basis of neuroconnectivity, Leuven, Belgium, May 4-6, 2006
- **Verhoeven K**.: Mutations in mitofusin 2 are a major cause for autosomal dominant axonal CMT neuropathy. VIB Seminar 2006, Blankenberge, Belgium, March 9-10, 2006

Progress Report of the Research Group of

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Developmental mechanisms patterning neuronal connectivity in the cerebral cortex.

The cerebral cortex is one of the most complex and important structures in our brain. In correlation with its elaborate functions, it is characterized by a huge diversity of neuronal phenoptypes, each cortical neuron displaying a specific pattern of connectivity. The mechanisms of formation of cortical networks have direct relevance to several diseases, such as epilepsy and mental disorders, as well as for the development of rationally designed cell therapies for neurological conditions.

The major research goal in our laboratory is to understand the mechanisms controlling the development of the cerebral cortex, from stem cells to neuronal networks and from mouse to man, by combining molecular and cellular approaches, both *in vivo* (using mouse transgenesis and in utero electroporation) and *in vitro* (using organotypic assays and embryonic stem cells).

We have summarized below the work achieved over the past year thanks to the Funding of the FMRE/GSKE, providing a link with our previously published work, as well as its perspectives in the future.

1. Multiple roles for ephrin/Eph genes in the development of the cerebral cortex.

We previously demonstrated that ephrin-A5 and its receptor EphA4 are required not only for the control of topographic specificity *within* the somatosensory area, but also unexpectedly for the *inter-areal* specificity of thalamocortical projections between the motor and the somatosensory systems (Dufour et al., 2003;Egea et al., 2005;Seibt et al., 2003;Vanderhaeghen and Polleux, 2004). Taking advantage of this system, we have now investigated in vivo the signalling mechanisms of neural mapping mediated by the EphA4 receptor, previously shown to control topographic specificity of thalamocortical axons in the mouse somatosensory system. Using axon tracing analyses of six knock-in mouse lines displaying selective mutations for the *ephA4* gene (obtained in collaboration with R. Klein, MPI Munich), we determined for the first time which intracellular domains of an Eph receptor are required for topographic mapping (Dufour et al., 2006). We obtained direct in vivo evidence that the tyrosine kinase domain of EphA4, as well as a tight regulation of its activity, are required for topographic mapping of thalamocortical axons, while non catalytic functional modules such as the PDZ binding motif (PBM) and Sterile-α motif (SAM) domain are dispensable. These data provide novel insight into the molecular mechanisms of topographic mapping, and constitute a physiological framework for the dissection of the downstream signalling cascades involved (Dufour et al., 2006).

In parallel we had generated a novel ephrin gain of function mouse model, enabling ectopic, patterned, expression of ephrin-A5 in the developing cortex. The analysis of this model, combined with our study of EphA7 knock-outs, enabled us to demonstrate an important role for ephrins in the control of brain size, through the unexpected regulation of apoptosis of neural progenitors (Depaepe et al., 2005;Depaepe and Vanderhaeghen, 2005). These findings suggest that ephrins, like neurotrophins, have evolved as pleiotropic factors that can control very different functions depending on the cellular context, which could have important implications in other aspects of developmental or stem cell biology, and in oncogenesis, as was recently confirmed by other groups

(Noren et al., 2006). We have followed up on these observations, focusing on the physiological impact of ephrin-mediated apoptosis in the patterning of different brain regions, and exploring the links between ephrin-dependent signals mediating apoptosis and axon guidance, both in neurons and in neural stem cells. On the one hand we have extended our observations by studying compound knock-out lines for ephrin-A2, ephrin-A3 and ephrin-A5 compound genes. Importantly we found in these mutants an even more robust appearance of exencephalic forebrain overgrowth previously observed in EphA7 knock-out mice (Depaepe et al., 2005), together with a reduction of apoptotic rates, not only in the cortex, but also in the ventral part of the forebrain, thus enabling us to conclude that ephrin-mediated apoptosis is a more general phenomenon during brain development (V. Depaepe and P.V., unpublished data). In addition we have obtained evidence that ephrin-dependent apoptosis is essentially observed on proliferating neural progenitors, while caspase 3 activation, but not cell death, is observed following ephrin stimulation of post-mitotic neurons. These data provide first insights about the cellular mechanisms of ephrin-dependent apoptosis, that seems to be highly dependent of the cellular context, which will be studied further using cortical progenitors and neurons in vitro.

2. Mechanisms upstream of ephrins during brain development.

A critical but still unsolved issue of forebrain development concerns the mechanisms responsible for the specification of distinct areal domains in the neocortex (Sur and Rubenstein, 2005). In this context, the early appearance of graded ephrin/Eph expression in several areas of the cortex (Vanderhaeghen et al., 2000) raises the question of how these gradients of expression are generated and controlled.

In vivo and in vitro approaches are being used to address this important question. First, we are generating mouse knock-in lines allowing expression of reporter genes (eGFP and PLAP) under the control of all regulatory sequences of ephrin-A5. In parallel we have developed an in vitro organotypic model combined with electroporation, in order to study the regulatory sequences responsible for the control of transcription of ephrin-A5 and EphA7. This system has recently enabled us to identify a 6 kb fragment of the EphA7 promoter, that seems necessary and sufficient to direct expression of a GFP reporter to cortical progenitors, and not to cortical neurons or cells of more ventral or caudal domains of the developing brain, which corresponds to the physiological pattern of expression of EphA7 at early stages of development (Depaepe et al., 2005). These findings will be followed up by transgenesis in vivo, while we wil test in vitro the involvement of candidate transcription factors, such as Neurogenin2 and Pax6.

3. Control of neuronal migration to the striatum and the cerebral cortex.

Work from several laboratories has demonstrated that in rodents most cortical GABAergic interneurons are generated in the ganglionic eminences (GE) in the ventral telencephalon, and subsequently migrate to the cerebral cortex through several streams of tangential migration (Marin and Rubenstein, 2001). Most interneurons destined to the cerebral cortex are generated within the medial GE (MGE), while the lateral GE (LGE) essentially generates neurons destined to

the basal ganglia. This dorso-ventral sorting of migrating neurons may have important functional and behavioural consequences, such as the balance of GABAergic innervation in the striatum and cortex.

We have recently set up organotypic assays combined with electroporation-based transfection to dissect the molecular and cellular mechanisms that control the migration of distinct populations of neurons to specific domains of the telencephalon: in this system, GE explants overexpressing eGFP (obtained from transgenic mice or following focal electroporation) are co-cultured with organotypic slices of developing telencephalon and the migration patterns of eGFP+ cells can be monitored over several days. These assays allow to recapitulate several important aspects of tangential migration, such as the dorso-ventral sorting of MGE vs LGE neurons that occurs in vivo. Using these in vitro assays, we have started to study the role of several candidate guidance factors including ephrins in the patterning of the migratory streams in the basal forebrain and cortex. We plan to follow up on these in vitro observations using in utero electroporation and appropriate mouse transgenic models, in order to test for the consequences of the early disruption of migration patterns on cortical function in mature animals.

4. Molecular mechanisms of neuronal specification using ES cell-derived neurons.

The molecular mechanisms of neuronal specification involve region-specific morphogens that play an instructive role to drive progenitors to particular cellular phenotypes. For instance in the the telencephalon, several transcription factors of the Homeodomain and Helix-Loop-Helix families are differentially expressed in distinct domains and cooperate to generate distinct neuronal types such as pyramidal neurons in the cortex, projection neurons in the striatum or diverse types of cortical and striatal interneurons (Schuurmans and Guillemot, 2002). However the identity of the genes acting downstream and upstream of these transcription factors, as well as the genetic programmes involved in the generation of the huge diversity of cortical projection neurons and interneurons remain largely obscure.

Recently we have developed an in vitro model of neural differentiation of embryonic stem (ES) cells to study the specification of cortical neurons (N. Gaspard and P.V., manuscript in preparation). Using a chemically defined medium devoid of any exogenous morphogen factors, we found that mouse ES cells cultured as monolayers spontaneously and efficiently (>80%) give rise to a population of neural precursors expressing regional markers indicative of a forebrain identity. When exposed to appropriate morphogen antagonists during their differentiation, in particular inhibitors of the *Sonic-Hedgehog* pathway, the fate of the ES cell-derived forebrain-like neural progenitors can be efficiently (>75%) directed to an identity corresponding to the cortical lineage, expressing Pax6, Tbr2, Emx1-2, Otx1-2 and Fox-G1 transcription factors.

ES cell-derived cortical-like progenitors subsequently differentiate into a stereotyped population of neurons, most of which (>75%) display all landmarks of cortical pyramidal neurons, including a glutamatergic phenotype and a pyramidal morphology. Most strikingly, ES cell-derived cortical neurons sequentially express a defined repertoire of markers that correspond to distinct subtypes

of cortical neurons, in a manner strikingly similar to the in vivo situation: cells expressing markers of Cajal-Retzius neurons are first being generated, follwowed by pyramidal neurons expressing markers of deep cortical layers, and eventually by neurons expressing markers of the superfical layers. Finally ES cell-derived cortical neurons can effectively integrate into postnatal cortical tissue, and display electrophysiological and synaptic activity profiles indistinguishable from excitatory cortical neurons.

Our results indicate that ES cells can be efficiently specified into cortical neural progenitors and pyramidal neurons in a chemically defined medium lacking any morphogen and containing only morphogen inhibitors, thereby surprisingly suggesting a « default pathway » of cortical neuron specification. This model of in vitro corticopoiesis recapitulates all milestones of cortical development observed in vivo and therefore constitutes an attractive and robust system to be used for the genetic dissection of the mechanisms of cortical neuron specification. It could be also used as a robust source of cortical neurons for pharmaceutical screens or pave the way for the rational design of cellular therapies of the cerebral cortex.

We have also started to implement similar methods to human ES cells to generate stereotyped patterns of pyramidal neuron specification. This could have a major impact for the design of cortical neuron replacement therapies, and in our understanding of human neuronal development. The use of human ES cell models will be also invaluable for our further studies on human cortical development.

5. Developmental basis of human-specific features in the cerebral cortex.

Although many aspects of brain development seem to be remarkably conserved throughout evolution, a number of neural features have undergone a considerable divergence in mammals, in particular in the forebrain. We therefore started a project focusing on the developing human brain, trying to reveal what are the specific developmental programmes underlying the emergence of human-specific features in our brain. This project already allowed us to demonstrate for the first time the involvement of ephrin/Eph genes in the development of the human brain, and to propose a new model for the emergence of a binocular visual system during mammalian evolution (Lambot et al., 2005).

In this frame we obtained recent evidence, in collaboration with the bioinformatic group of D. Haussler (HHMI UCSC) that HAR1 (*Human accelerated Region 1*), a novel non coding RNA gene that is highly conserved throughout amniotes but contains among the most highly divergent sequences in the human lineage (Pollard et al., 2006). We were able to show that HAR1 is strongly expressed in the human embryonic neocortex, in particular in Cajal-Retzius (C-R) neurons, a transient cell population, particularly prominent in primates, that plays a major role in the control of cortical paterning from mice to man (Amadio and Walsh, 2006). In addition HAR1 displays a novel and highly conserved secondary structure of unknown function (Pollard et al., 2006), that makes it an attractive candidate for regulation of gene expression, similarly to miRNAs.

Given its potential involvement in the development and evolution of the cerebral cortex, we are now focusing on the function of HAR1. On one hand we are undertaking a gain-of-function approach, using electroporation of human and mouse HAR1 expression constructs in mouse embryonic brain (by *ex vivo* and *in utero* electroporation, both used routinely in the lab), looking for alterations in neuronal migration, differentiation, and connectivity. Particular attention will be focused on potential differences between effects of mouse and human HAR1. On the other hand, we are developing in vivo transgenic approaches to generate HAR1 gain and loss of function. We have started to generate knock-out mice for the HAR1 gene using classical homologous recombination in ES cells, targeting the ultraconserved domain of HAR1 present in the mouse, and replacing it by a Cre- excisable selection cassette. In parallel we are generating a gain of function line by knocking a condtional (Cre dependent) HAR1 expression construct into the ROSA26 locus (Belteki et al., 2005). Mice derived from recombined HAR1/ROSA26 ES cells will allow robust and conditional expression of HAR1 in vivo following appropriate crosses with Cre expressing mice such as Emx1, FoxG1 and Dbx1 Cre mice. Both gain- and loss-of-function conditions will be analyzed, looking for potential changes in neural proliferation, migration, specification, and connectivity using techniques routinely used in the lab (Depaepe et al., 2005;Dufour et al., 2003). Together with the study of HAR1 in neurons derived from human ES cells, this work may enable us to uncover the biological function of this gene that may have played a crucial role in human brain evolution.

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Progress Report of the Research Group of

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Interactions between areas investigated using awake monkey (f)MRI.

1. Topographic specific functional interactions between frontal eye-fields and visual cortex.

It is well-known that neurons in extrastriate and even striate visual cortex modulate their firing rate, depending on the direction of attention of the subject with respect to the receptive field of the neuron. Furthermore, a clear correlation has been suggested between such modulated neuronal activity at attended locations and corresponding benefits at behavioral level. However, the neural mechanisms that give rise to these effects are poorly understood. Two possible mechanisms have been suggested: a bottom-up mechanism in which salient stimuli in the visual scene automatically 'attract' attention without the need of endogenous signals. Secondly, it is widely recognized that voluntary and hence endogenous attentional 'source' signals emerge in higher level areas within a fronto-parietal network. Feedback signals from these areas to lower level sensory areas are thought to modulate incoming sensory information through feedback and lateral connections. More in particular, it has been suggested that the a prefrontal region involved in the generation of purposeful eye-movements (frontal eye fields , or FEF) may play an important role as attentional top-down source to modulate incoming sensory information. In this study, we aimed to specifically examine whether the latter region is able to modulate visually driven activity in a retinotopic specific manner.

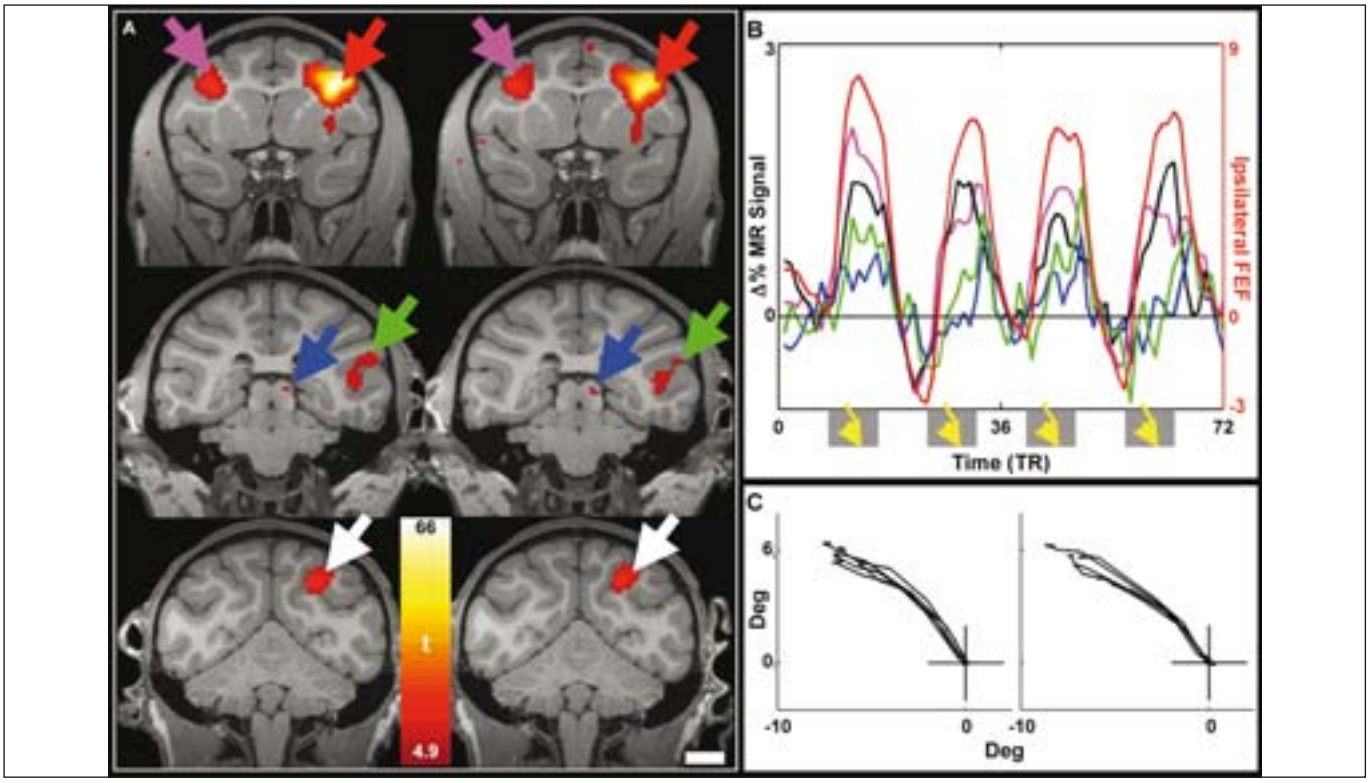

Fig. 1. in-vivo tractography by microstimulation of right FEF. The figure illustrates the accuracy, precision and repeatability of the technique.; **(A)** Coronal slices showing t-scores of fMRI activity ($p < 0.05$, corrected) induced by microstimulation (arrows: red = ipsilateral FEF, pink = contralateral FEF, blue = SC, green = STP, white = LIP); columns represent test & retest - distinct experiments collected one month apart. Scale bar indicates 10 mm. **(B)** Time courses of percent MR signal change sampled from the left member of each pair in (A). Color coding identifies sample location, black represents the white sample, the red trace is plotted on the right axis and the grey bars indicate stimulation epochs. **(C)** Example eye traces recorded in MR scanner, separated by one year, showing saccades evoked by stimulation of the same electrode.

During last year's experiments, we started to study the functional role of connections arising in the FEF using a combination of electrical microstimulation and fMRI (EM-fMRI) in awake behaving monkeys.

This novel combination of techniques proved to be an excellent tool to trace functional connections in-vivo. As shown in Fig 1, regions which are known to be connected anatomically with the FEF showed enhanced fMRI activity during microstimulation of the FEF in avery precise, accurate, sensitive, and repeatable manner (spatial correlation between activation maps of two different experiments can be as high as 0.84). Secondly, we also showed that the artificially increased output of the FEF, modulates visually-driven activity in occipital cortex as long as the visual stimuli are centered over the stimulated FEF movement fields (see last years report). This year, we extended these experiments to two animals and we were able to confirm the results of the first animal.

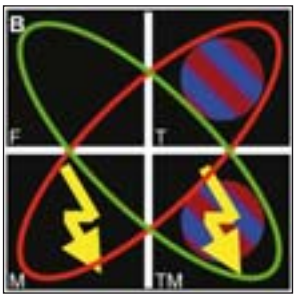

Fig. 2. Factorial design

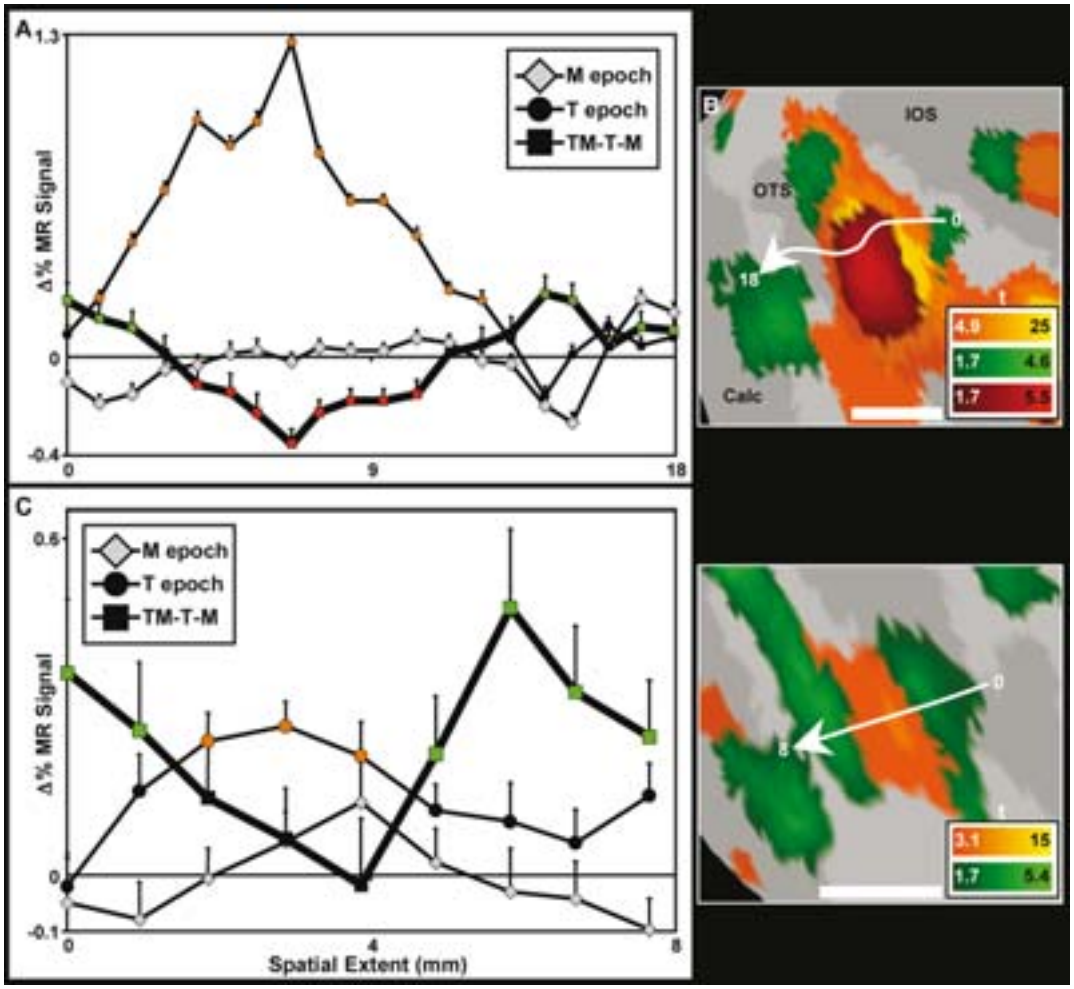

Fig. 3. fMRI activity along a path parallel with cortical surface (occipital cortex, two animals); **(A)** From subject T, % Change in MR signal with respect to the baseline F epoch during M (M, 'microstimulation') and T (T, 'Target' = visually driven activity) epochs, and for the interaction contrast, TM-T-M. Color code indicates significant positive interactions (green) or negative (red) interactions in the factorial design of Fig 2. (B). Error bars = s.e.m.

(B) Location of cortex sampled in (A); (orange = visually-driven activity, $p < 0.05$ corrected; green / red = visually-driven region and positive / negative interaction, $p < 0.05$ conjunction). Arrow indicates direction and approximate location of sampling; scale bar indicates 5 mm. **(C)** data from subject D. **(D)** Flattened ventro-occipital patch, showing location of cortex sampled in (C);

Moreover, we now investigated in detail the topographic interactions between visually driven activity in occipital cortex and electrical subthreshold microstimulation of the FEF. To this end we used a factorial design (See fig 2, T = Target, or visually driven activity, M = microstimulation of the corresponding movement field of the FEF). We placed visual stimuli (colored high-contrast gratings) within movement fields of the FEF that were either electrically stimulated (using subthreshold stimulation currents, thus without evoking an eye-movement), or without stimulation. The results show that: 1) positive as well as negative interactions between EM and visually driven activity can be found in visual cortex. 2) The positive interactions between EM and FEF are largely restricted to those visually driven regions that are not yet optimally driven in the absence of EM. 3) Negative interactions are relative less-widespread than positive interactions.

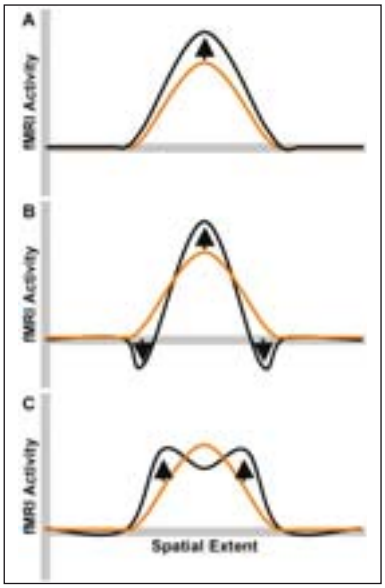

Fig. 4. Three possible models for enhanced visually-driven fMRI activity by FEF microstimulation. **(A)** Peak enhancement – voxels at the center of the representation become more active; **(B)** Peak enhancement with surround suppression – central voxels become more active, perimetric voxels become less active; **(C)** Edge enhancement – perimetric voxels in the shoulder of the representation become more active, with little change or a slight decrease in the peak voxels.

Surprisingly though, they are mainly present at those locations where visually-driven activity is highest in the absence of EM (see fig 3). Detailed eye-movement analysis showed no significant interaction between number of saccades, or distribution of the X and Y position of the eyes and the different experimental conditions. Thus eye-movements cannot explain the observed results.

These data show a **causal** relationship between the artificially increased output of the FEF and topographic-specific modulated visually-driven activity in visual cortex. Moreover, our data suggest that the increased output of specific FEF sectors results in enhanced visual representations 1) by boosting the fMRI activity of those cortical sectors that were not yet optimally driven by the visual stimulus (in the absence of 'top-down' input), and 2) suppressing the fMRI activity of those sectors that were already optimally driven (favoring the model presented in Fig 4C). Thus, spatially-specific increased top-down input in visual cortex will lead to topographic-specific activation of more cells to process incoming visual information, rather than up-modulating the most active cells. Hence, these data suggest that the FEF does indeed contain the hardware to modulate visually-driven activity in an attention-dependent manner. This will be tested in the future by combining EM-fMRI with monkeys performing a spatial attention task.

2. The effect of cerebral glucose metabolism on diffusion MRI

Diffusion MRI is powerful diagnostic tool for a wide variety of pathologies (stroke, schizophrenia, Huntington's disease, Alzheimer's disease). Surprisingly, however, despite the widespread application of diffusion MRI, the underlying mechanisms for diffusion contrast in cerebral matter remain largely unknown. Therefore, it is also unknown which specific biological mechanisms (metabolism, blood flow, neuroarchitecture, etc) lead to diffusion changes in pathology. In clinical diffusion MRI, the two scalar measures of diffusion are the apparent diffusion coefficient

(ADC) and the fractional anisotropy (FA) of cerebral tissue. The ADC represents the magnitude of the diffusion whereas the FA is a measure of the anisotropy of the diffusion. In this study, we investigated whether cerebral glucose metabolism (CGM), and/or processes associated with CGM, contributes to the ADC and FA as measured by diffusion tensor imaging (DTI).

A magnetic resonance (MR) compatible cooling system was built to reversibly deactivate focal cortical cerebral glucose metabolism (45 minute deactivation times) in small portions of primary visual cortex of two rhesus monkeys.

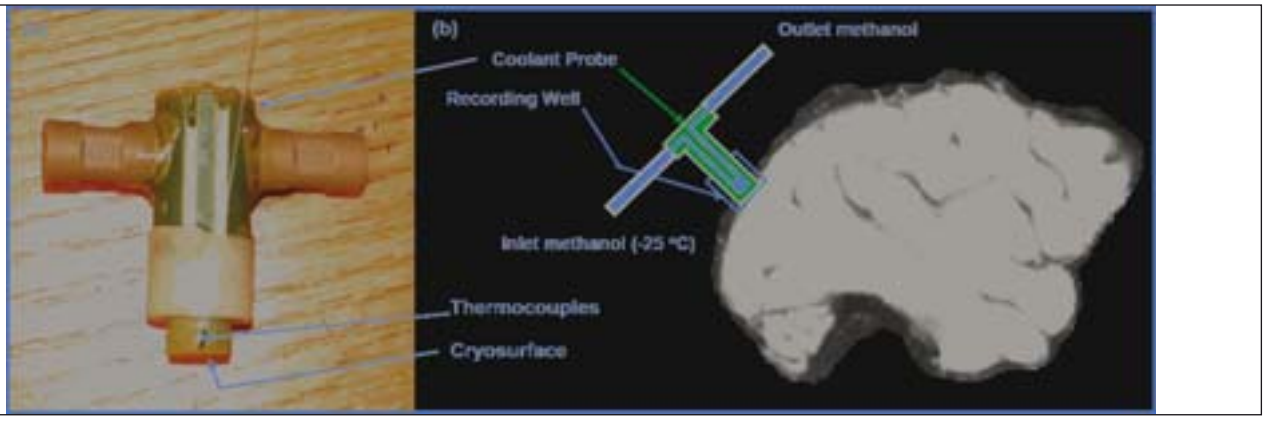

Fig. 1 schematic of MRI-compatible cooling probe

The accuracy and precision of the MRI-defined temperature maps were first quantified in ex-vivo bovine muscle by comparing the temperature calculated from the MR images to a magnet-compatible thermocouple placed immediately under the coolant probe. Temperature maps were calculated from the MR thermometry scans using the difference in phase between successive time points:

$$\Delta\varphi = 2\pi.\alpha.\gamma.TE.\Delta T + \Delta\varphi_{drift}$$

where $\Delta\varphi$ is the change in phase, α is the thermal coefficient (0.01 ppm/°C), TE is the echo time of the pulse sequence, and ΔT is the temperature change (after a correction to account for the linear phase drift $\Delta\varphi_{drift}$, which occurs even in regions where the temperature does not change).

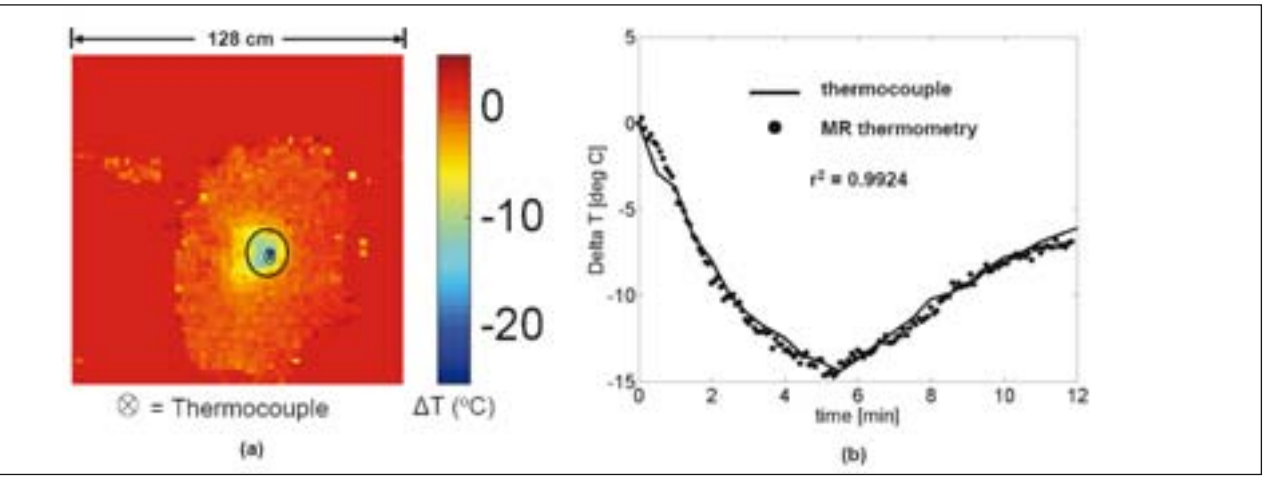

Fig. 2 Comparison of MR-thermometry measurements with MRI-compatible thermocouple

Fig. 2a shows a sample MR-defined temperature map of a slice under the cooling probe. Fig. 2b shows the comparison of the MR thermometry values (averaged from four voxels in the first slice under the probe) with the thermocouple measurements. The precision of the temperature maps at a resolution of 2.1 x 2.1 x 1 mm^3 was ± 1.8 °C and the accuracy of the MR temperature values were within ± 0.8 °C of the thermocouple measurements. The high correlation coefficient between the MR thermometry maps and the thermocouple (r^2 = 0.99) illustrates the robustness of the phase difference method for calculating temperature. The accuracy of the temperature maps in subsequent in-vivo cooling experiments in monkeys was ± 1 °C at a resolution of 2 x 2 x 2 mm^3.

Experimental Design

Under anesthesia, DTI was acquired during warm conditions while warm methanol was pumped through the cooling system. This was done to ensure that any measured change in diffusion was not due to artifacts created by flow. During different experiments, primary visual cortex was cooled to different cortical temperatures (either above or below the metabolic cut-of temperature of 20 deg). During the initial cooling period (~12 minutes), MR temperature maps were acquired until a constant cortical temperature was reached as measured by an MR-compatible thermocouple placed immediately under the probe on the surface of the brain (± 1 °C). The temperature distribution in the brain was kept constant over rest of the cooling period (20 minutes) when DTI images were acquired. Finally, the brain temperature was allowed to recover slowly while MR temperature maps were acquired (~8 minutes).

In-vivo cooling: effect on ADC and FA maps

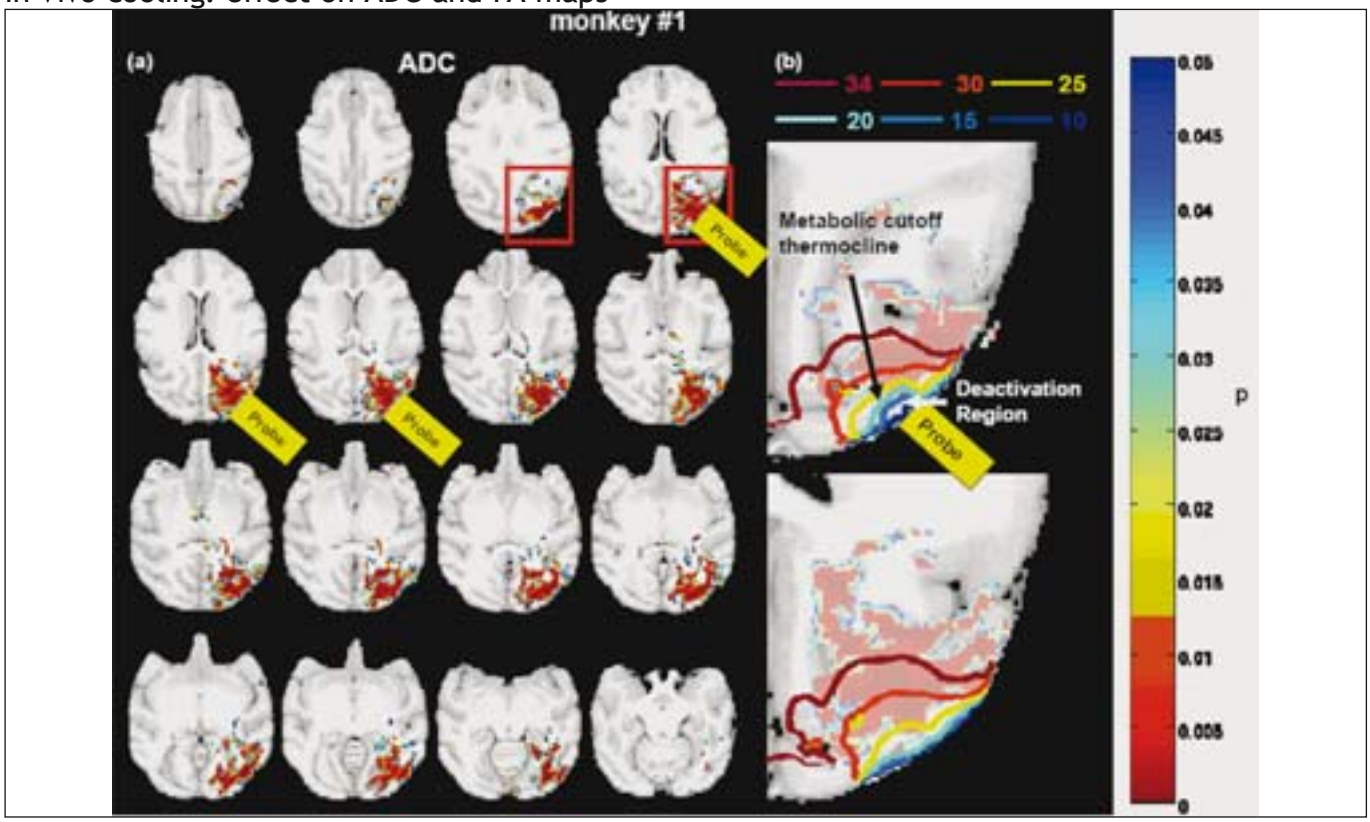

Fig. 3 Significant changes in ADC maps after reversible deactivation of V1 (non-parametric permutation (NPPT) tests, and overlaying thermoclines based on thermometry)

MRI-based thermometry was used to dynamically acquire 3-dimensional temperature maps of the brain in order to define the region and degree of deactivation during anesthetized experiments. Only in those deactivation trials in which brain tissue was cooled below the metabolic cutoff (20 °C), i.e. the temperature below which cerebral metabolism ceases, we observed changes in the ADC (10% - 25%, $p < 0.001$). Importantly, changes in ADC occurred in brain regions at a distance from the cooling probe where no temperature changes were measured (12-20%, $p < 0.001$). This is strong evidence that the changes in the ADC are not due to the effect of temperature on diffusion alone. No statistical significant changes in fractional anisotropy (FA) were observed during metabolic deactivation (NPPT-testing), neither under the probe nor at a distance from the probe. Also more sensitive ROI-analyses showed clear changes in ADC (and not for FA) for ROI's close to the cooling probe (and where temperature was unaffected) –see Fig. 4.

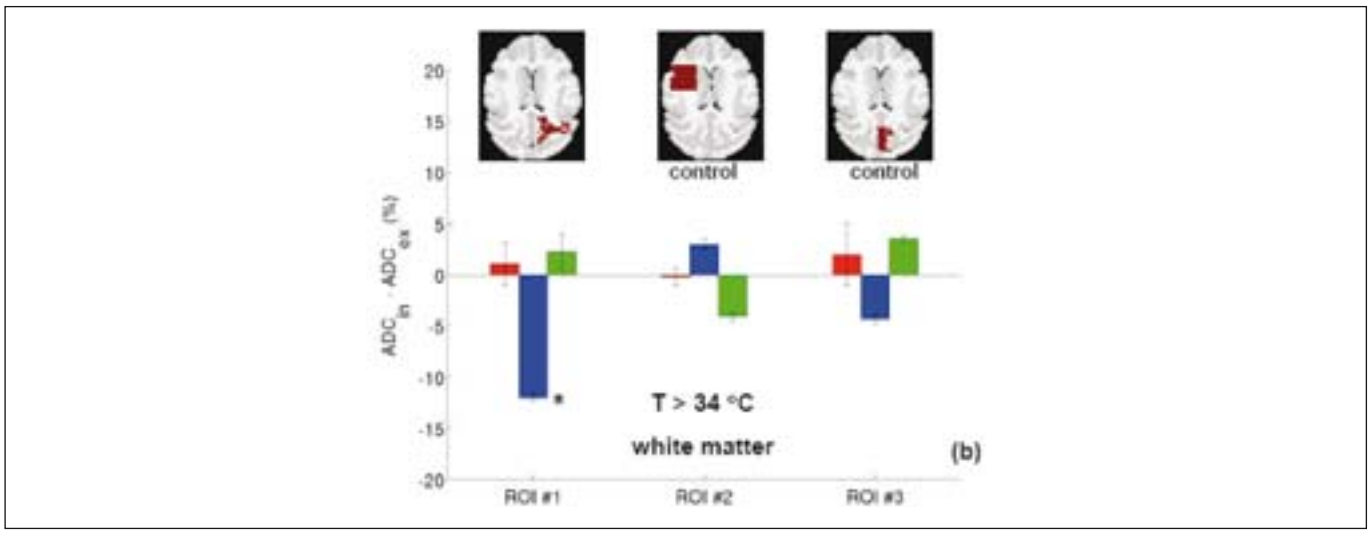

Fig. 4. ROI-analysis of ADC-changes in ROI's in which the temperature was not affected by cooling. Only the first ROI showed a significant change in ADC (not ROI's at a far distance or ROI's at an equal distance from the probe compared to ROI 1)

We can conclude ADC but not FA maps are significantly altered after reversible deactivation of cerebral cortex, even in regions in which there is NO change in temperature. We were able to measure 3D thermoclines with a precision of ± 1 °C.

We suggest that the effects of cooling on the ADC maps can be explained by decreased cerebral glucose metabolism. Cooling results in a decrease in the deoxyglucose uptake and action potential cannot be generated below 20 °C. In our experiments we observed only ADC changes at distant sites when the temperature under the cooling probe was below the metabolic cutoff of 20 °C and NOT above 20 °C.

Fast axonal transport is one mechanism affected by cerebral glucose metabolism that could account for the distant ADC changes. Fast axonal transport is responsible for the transport of proteins to and from the neuron through the axons. The distribution of velocities in the axon could lead to changes in diffusion coefficients as measured by diffusion MRI. Also, fast axonal transport is directly regulated by ATP. Deactivating cerebral metabolism affects the production of ATP and the regulation of fast axonal transport in axons leaving the deactivated region.

The ADC decreases observed during this experiment explains at least some of the marked decrease in the ADC during ischemia (20% to 50%) and post-mortem studies (~ 40%), and upon digestion of the microtubule network required for axonal transport. FA changes would not be expected to change in this experiment because of the short deactivation period. Changes in the FA in stroke and lesion studies, have been shown to occur over a long time period (days) and related to other neural processes like Wallerian degeneration suggesting that FA is not directly related to cerebral metabolism.

In the near future the MR-compatible RD cooling system will also be used in combination with fMRI. RD can be used in conjunction with fMRI to deactivate a known region and measure the functional consequences in rest of the brain.

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Progress Report of the Research Group of

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The human 5-HT7 receptor: a new link and potential therapeutic target for Alzheimer's Disease?

Introduction

Alzheimer's Disease (AD) is the most common neurodegenerative disorder of the central nervous system, characterized by progressive impairment of memory and cognition. It is the most frequent form of dementia found in the elderly [1]. The prevalence of AD is 5-10% at 65 years of age and increases to 20-50% by the age of 85. By 2050, the population over 65 years of age will approximately double, which translates to well over 20 million patients with AD in the United States and Europe.

Two types of protein aggregates in the brain characterize AD: the intracellular neurofibrillary tangles (NFT), consisting of hyperphosphorylated tau and the senile plaques, which are largely composed of the extracellular deposition of amyloid-β (Aβ), a 40-42(43) amino acids polypeptide. Aβ is generated from Aβ precursor protein (APP), which belongs to a larger gene family including amyloid precursor like protein 1 and 2 (APLP1 and APLP2). All three proteins are type I, integral membrane proteins with a large glycosylated extracellular region (N-terminal), one transmembrane helix and a short C-terminal cytoplasmic tail and are all processed by proteases. First, they are cleaved in their extracellular part by either the α- or β-secretase resulting in soluble extracellular domain (sAPPα/β or sAPLPα/β) and an intermediate membrane-bound fragment (C83 and C99, respectively). The latter is then further processed by the γ-secretase. In case of APP, sequential proteolytic cleavage by β- and γ-secretases, results in the secretion of Aβ [2-4].

Although the decline in cognitive functions seen in AD might be largely related to cholinergic dysfunction arising from disruption of basal forebrain cholinergic pathways [5, 6], a disturbed balance between several neurotransmitter systems has been implicated in the pathogenesis in AD [6-8]. Amongst this, serotonin (5-HT) seems to be playing a pivotal role [9-13]. Serotonin is an important neurotransmitter involved in diverse physiological processes such as sleep, sexual behaviour, food intake, mood regulation and certain cardiovascular and gastro-intestinal functions. These diverse physiological effects of 5-HT are mediated through 14 distinct mammalian 5-HT receptor subtypes. With the exception of the 5-HT3 receptors, all belong to the family of 'G protein-coupled receptors' (GPCRs) (for review, see [14, 15]). This diversity is even more amplified by post-genomic modifications such as alternative splicing and editing of the mRNA.

The most recently identified serotonin receptor is the 5-HT7 receptor. This receptor subtype is involved in circadian rhythm, REM sleep, depression, hippocampal signalisation, memory, thermoregulation, migraine and endocrine regulation (reviewed in [16, 17]). Alternative splicing of the 5-HT7 mRNA gives rise to different isoforms, namely 5-HT7(a), (b), (c), (e) in rat and 5-HT7(a), (b), (d) in humans [18-20]. These splice variants differ in the length of their intracellular C-terminal tail and in the number of potential phosphorylation sites within this region. This suggests variation in signalisation and regulation. To further investigate these potential differences, we decided to search for proteins interacting with these different C-terminal regions. Therefore, we performed a yeast two-hybrid screening with the C-terminal end of the human 5-HT7(a) receptor

as a bait and an adult-human brain cDNA library as prey. This led to the identification of several interesting possible interaction partners, of which the human β-Amyloid Precursor like Protein 1 (APLP1) was one. This interaction indicates an innovative link between the 5-HT7 receptor and Alzheimer's Disease.

Research: current status and prospectives

Interaction studies

To validate the interaction observed in the yeast two-hybrid experiments, we first investigated whether both proteins interact in eukaryotic cells. After transient transfection in COS cells, we were able to demonstrate co-immunoprecipitation of APLP1 with all three 5-HT7 receptor isoforms. Follow-up experiments in COS cells revealed that the 5-HT7 receptors also interact with APP and APLP2. For APP and APLP1, the interaction with the 5-HT7(a) receptor was also confirmed in the mouse neuroblastoma cell line HT22 (Figure 1). This cell line not only exhibits a high endogenous expression of APP, but we could also demonstrate the presence of 5-HT7 specific mRNA by RT-PCR and we are currently optimising the conditions to reveal the presence of the 5-HT7 receptor at the protein level. To exclude that these interactions are not due to transient overexpression of both proteins we made use of CHO cells, stably expressing APP (gift from Drs. D. Walsh and D. Selkoe, Department of Neurology, Harvard Medical School and Center for Neurologic Diseases, Boston, Massachussetts). After transient expression of the 5-HT7(a) receptor in these cells, we could also co-immunoprecipitate APP with the 5-HT7(a) receptor.

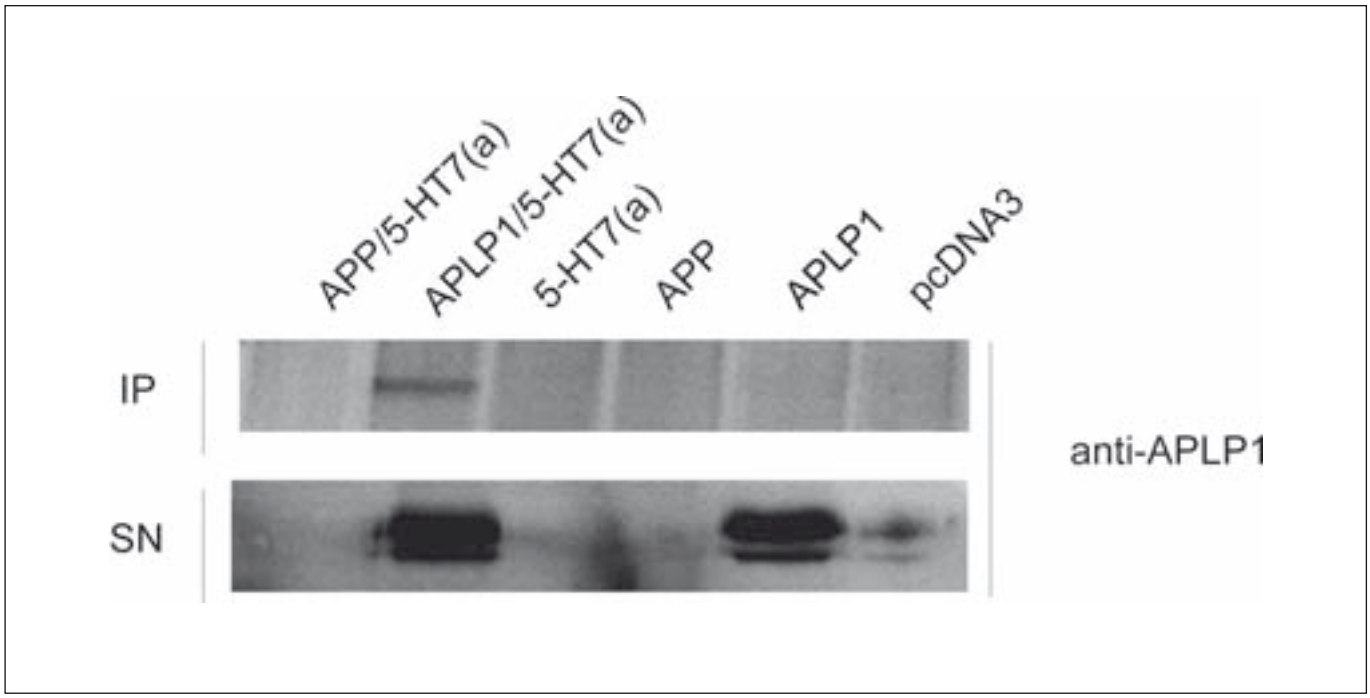

Fig. 1: Co-immunoprecicipitation between the HA.5-HT7(a) receptor and APLP1 in HT22 cells.

HT22 cells were transiently transfected with the HA.5-HT7(a) receptor and human APLP1 alone or in combination. Immunoprecipitation was performed with a mouse antibody directed against the HA-tag. The immunoprecipitated proteins were revealed with anti-APLP1 (anti-CT11).

However, the 5-HT7 receptors and the APP proteins are both transmembrane proteins and thus firmly attached to the plasma membrane of eukaryotic cells. This still implicates the possibility that these proteins co-immunoprecipitate without direct interaction, but rather as a consequence of their anchorage in the plasmamembrane. To further confirm that these proteins are genuine interaction partners, we decided to follow an in vitro GST pulldown approach. After immunoprecipitation of the GST proteins with glutathione beads, we detected a clear signal for APP, APLP1 and APLP2 on the beads with the C-terminus of the 5-HT7(a) receptor, but not on the beads with the GST protein alone (Fig. 2). All together, these data strongly suggest a direct interaction between the APP proteins and the 5-HT7 receptors.

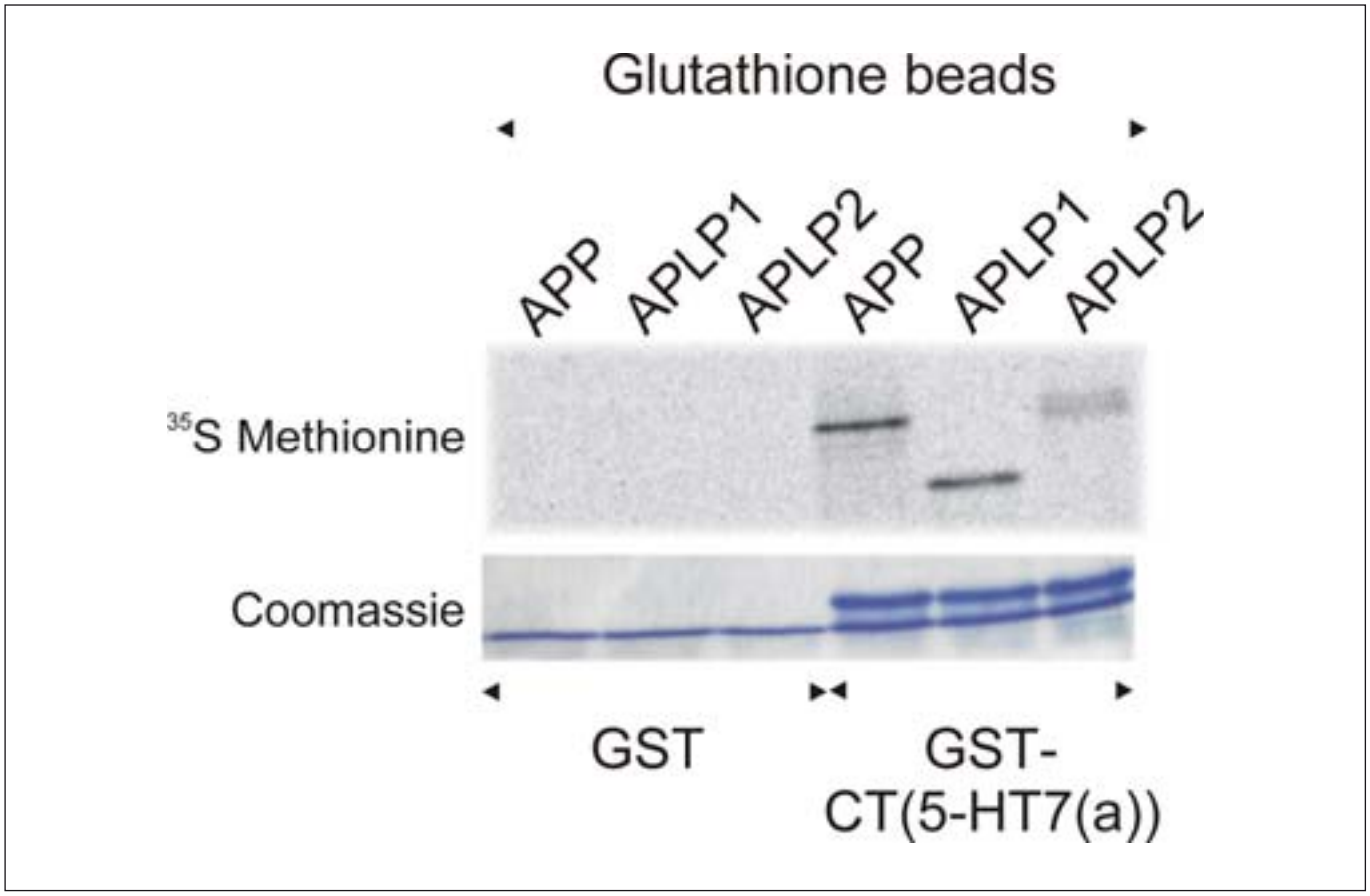

Fig. 2: GST pulldown of GST-CT(5-HT7(a)) with APP, APLP1 and APLP2.

APP, APLP1 and APLP2 were produced by in vitro transcription/translation in the presence of ^{35}S Methionine. These proteins were then added to the GST-CT(5-HT7(a)) fusion protein and to GST protein (as a negative control). After immunoprecipitation with glutathione beads, proteins were separated by SDS-PAGE and visualised using a PhoshphorImager.

In the yeast two-hybrid screening, we used the intracellular C-terminal part of the 5-HT7(a) receptor as a bait. Therefore, it is reasonable to assume that the interaction with the APP proteins also occurs via their intracellular C-terminal part. However, we could not co-immunoprecipitate two different C-terminal deletion mutants of APLP1, namely C51 en C60 (provided by Dr. D. Walsh) with the 5-HT7(a) receptor. A decreased stability and incorrect cellular localisation of these fragments

could be the reason for this lack of interaction. To avoid this topological problem, we want to use an 'in vitro' GST pulldown approach. We currently have five C-terminal APLP1 deletion mutants available (kind gift from Dr. Weber, University Hospital Schleswig-Holstein, Kiel, Germany, [21]) which will be used to narrow down the domain of APLP1 responsible for interaction. To identify to domain involved at the receptor side, we generated several 5-HT7 receptor deletion variants in which various parts of the C-terminus and the third intracellular loop have been deleted, and which will be used in co-immunoprecipitation experiments. Recently, the group of Weber et al., reported that APLP1 interacts with the α2-adrenergic receptors but not with the vassopressin V2 receptor [21]. Alignment of the amino acid sequences of the intracellular tails of these different receptors (Fig. 3) revealed the presence of a subset of identical amino acids. We are currently generating specific point mutations to identify which of these amino acids is/are essential for the interaction observed.

Fig 3.: Alignment of the amino acid sequences of the C-terminal tails of α_{2A}AR, α_{2B}AR, α_{2C}AR, 5-HT7(a)R, V2R, β2AR.

Identical amino acids are indicated in red ; highly homologous amino acids are indicated in yellow and homologous (structural and functional) amino acids are indicated in brown.

As literature indicates, the interaction of APLP1 is not restricted to the 5-HT7 receptor. In this respect, we will certainly investigate potential interaction with the 5-HT4-, 5-HT2A- and 5-HT2C-receptors as it has been shown that stimulation of these receptors influences the proteolytic processing of APP, although no direct interaction was reported. The cDNAs and cell lines inducibly expressing these receptors are available within the group [22, 23].

Co-localisation studies

An aspect of normal APP physiology is the role of APP in the axonal transport of vesicles. The cytoplasmic domain of APP apparently binds to the kinesin light chain thereby acting as the link between the transport vesicle and the kinesin motor complex [24]. Further to this, it has been reported recently that interaction with APLP1 alters the localisation of the α2A-adrenergic receptor. Therefore it is of interest to investigate whether the observed interaction between the 5-HT7 receptors and the APP-family of proteins has an influence on receptor tranport and/or receptor localisation. Our recent data indicate that we have a substantial degree of co-localisation between the 5-HT7(a) receptor and both APP and APLP1 in COS (Fig 4.) and HT22 cells (Fig 5.).

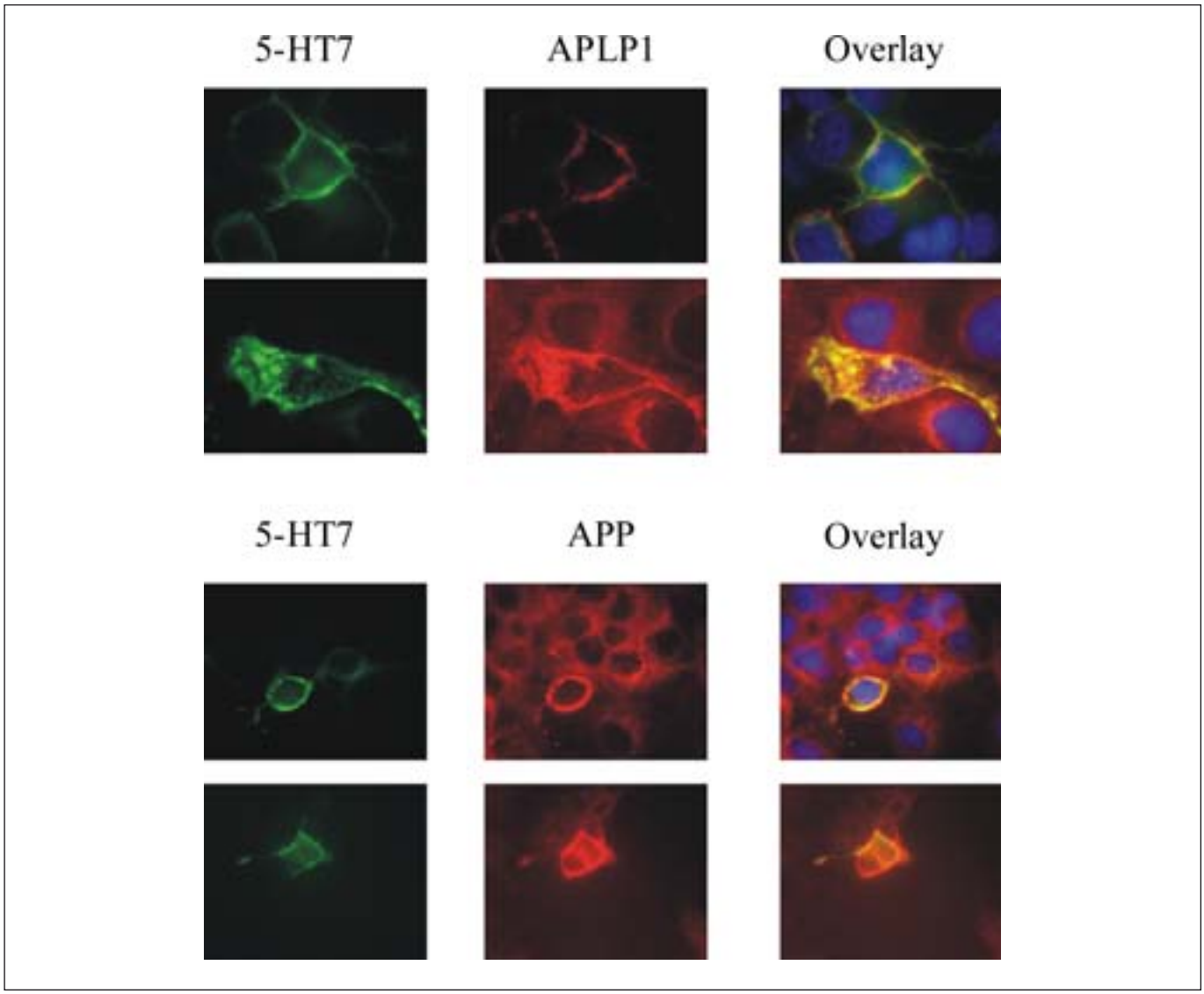

Fig. 4: Co-localisation studies between the c-myc.5-HT7(a) receptor and APP/APLP1

COS cells were transiently transfected with the c-myc.5-HT7(a) receptor and either APP or APLP1. Paraformaldehyde fixed cells were incubated with both primary antibodies (mouse anti-c-myc and rabbit anti-APP/APLP1) overnight. Subsequently, the samples were incubated with a combination of Alexa 488 (green) labelled anti-mouse antibody and an Alexa 594 (red) labelled anti-rabbit antibody. Finally, the coverslips were incubated with DAPI to stain the DNA.

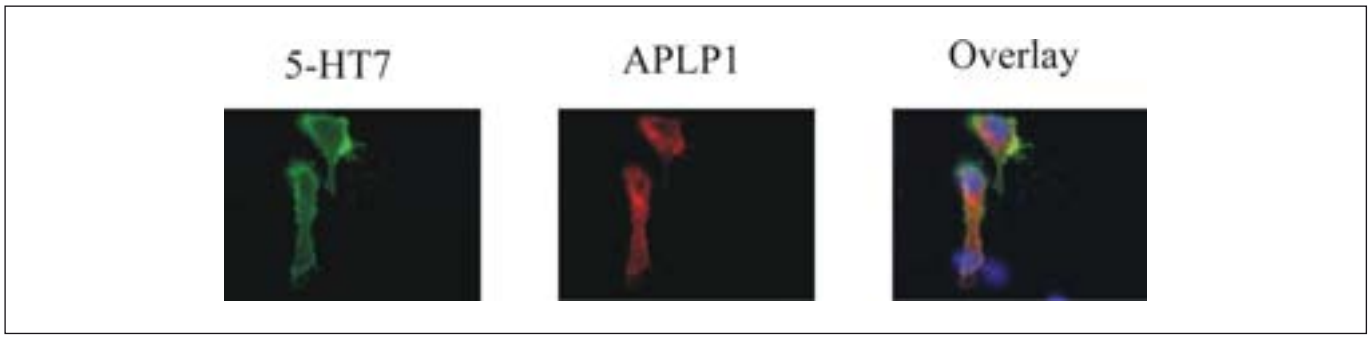

Fig. 5: Co-localisation studies between the HA.5-HT7(a) receptor and APLP1

HT22 cells were transiently transfected with the HA.5-HT7(a) receptor and APLP1. Paraformaldehyde fixed cells were incubated with both primary antibodies (mouse anti-HA and rabbit anti-APLP1) overnight. Subsequently, the samples were incubated with a combination of Alexa 488 (green) labelled anti-mouse antibody and an Alexa 594 (red) labelled anti-rabbit antibody. Finally, the coverslips were incubated with DAPI to stain the DNA.

We will extend our co-localisation studies to all 'APP family members' and the different receptor isoforms and attempts are currently ongoing to confirm the results in more relevant cell systems and with the endogenous proteins. We have several immortalized neuronal cell cultures in the lab. Our immunofluorescence data indicate that they all express APP and for a subset we also observed clear expression of APLP1 (Fig 6.).

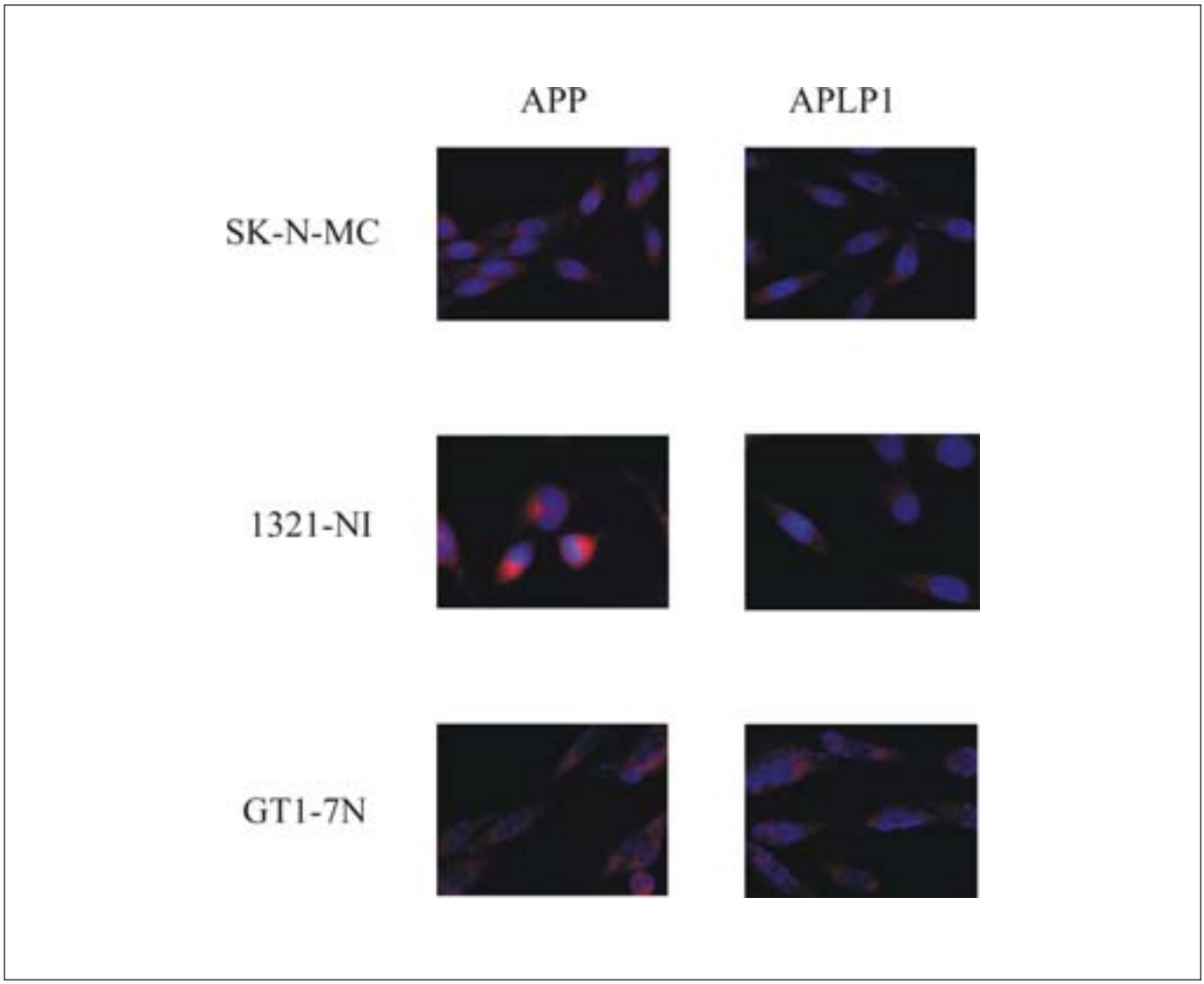

Fig. 6: Immunofluorescence studies of endogenous APP and APLP1 in immortalized neuronal cell cultures.

Several immortalized neuronal cell cultures were fixed with paraformaldehyde and incubated with a primary antibody against either APP or APLP1. Subsequently, the samples were incubated with an Alexa 594 (red) labelled anti-rabbit antibody. Finally, the coverslips were incubated with DAPI to stain the DNA.

For a number of these cell lines, our RT-PCR data indicate that they also produce 5-HT7 receptor specific mRNA. So far, we were not able to demonstrate the endogenous presence of the 5-HT7 receptor at the protein level, using the commercially available antibodies. We recently generated our own 5-HT7 receptor specific antibody and data on overexpression indicate that the antibody has a higher affinity as compared to the commercial ones. We hope that this antibody will allow us to detect endogenously the 5-HT7 receptor, which then should enable us to perform co-localisation studies (as well as the interaction studies described above) under physiologically relevant conditions. For all co-immunoprecipitation and co-localisation experiments, a comparison will be made between a receptor in resting state (- 5-HT), a stimulated receptor (+ 5-HT or a specific agonist, e.g. AS 19) and a situation in which the intrinsic constitutive activity is blocked (+ antagonist, e.g. SB269970).

By comparing the trafficking of wildtype receptors with receptors in which the APP-interacting domain has been deleted (see above), we should be able to study whether the interaction is necessary for a correct trafficking of the receptor. We already constructed various functional GFP-variants of the 5-HT7 receptor isoforms, which should allow us to follow this process in 'real time'.

Influence on 5-HT7 receptor signalling

Activation of the 5-HT7 receptor results in an increase in cAMP levels. Evidence exists that the activity of Gs-proteins is diminished in the affected regions of AD brains [25]. Therefore, we will investigate through direct cAMP-analysis (ELISA, RIA) or indirect assays (cAMP reporter genes) whether the interaction of the 5-HT7 receptor with APP/APLP1 affects this signalling cascade. Also the effect on the 5-HT7 receptor mediated activation of Erk1/2 [26, 27] will be investigated.

Role in APP processing

It has been shown that activation of 5-HT receptors can influence processing of APP. Stimulation of 5-HT4, 5-HT2A and 5-HT2C receptors leads to an increased secretion of the non-amyloidogenic soluble APP (sAPPα) [28-30]. As α-secretase hydrolyzes APP at Lys16-Leu17 within the Aβ sequence, stimulation of this non-amyloidogenic pathway not only precludes the formation of Aβ, but also generates a neurotropic sAPPα. Stimulation of these receptors might therefore be beneficial for the treatment of AD, which is in agreement with a recent study showing that specific activation of the 5-HT4 receptor inhibits secretion of Aβ and increases neuronal survival [31]. In contrast, another recent study shows that activation of the β2-adrenergic receptor and the δ-opioid receptor stimulates γ-secretase activity and accelerates Aβ formation [32]. As highlighted in Fig. 3, the C-terminus of the β2-adrenergic receptor shares some of the amino acids, which we believe could be involved in the interaction. Therefore, we are currently investigating whether activation of the 5-HT7 receptor results in either an increased production of sAPPα or in contrast leads to an upregulation of Aβ-production. For the detection of sAPPα, we already tested successfully domain-specific antibodies and worked out the conditions for efficient transfection of HT22 cells. For Aβ-production, commercially available ELISA-systems will be used. For the 5-HT4 receptor it has recently been shown that this increase production of sAPPα process is cAMP-dependent and Epac-mediated [33]. Given the fact that the 5-HT7 receptor makes use of Epac to activate Erk1/2

in hippocampal neurons [34], the possible role of Epac in the above mentioned experiments will be studied. This will be done through the use of dominant negative and constitutively active forms of Epac, which recently became available within the research unit.

The C-terminal fragment of the β-amyloid precursor protein produced after cleavage by γ-secretase, namely APPct or AICD, has been shown to form a multimeric complex with the adaptor protein Fe65 and to regulate transcription (e.g KAI-1 and GSK3β) through the recruitment of the histone acetyl transferase Tip60 [35]. If the 5-HT7 receptor influences APP processing, the role of interacting proteins such as Fe65, Tip60 will be studied with special interest in potential chromatin-modulating properties of Tip60. Therefore we will use techniques such as nucleosome positioning and chromatin immunoprecipitation assays, for which we have the expertise available within the research unit [36, 37].

Studies using knockout cells

For some experiments, we will try to confirm the data acquired by using cell lines where the 'APP family of proteins' is absent. The APLP1 knockout mice only display a modest postnatal growth retardation. APP loss-of-function leads to relatively mild neurological or behavioural deficits. The combined knockout mice display growth retardation problems and early postnatal lethality, which suggests that there is redundancy in the function of these proteins [38]. It is our aim to collaborate with the group of Dr. Müller (Department of Neurochemistry, Max-Planck-Institute for Brain Research, Frankfurt, Germany) and to use neuronal cells of the single knockout mice to study the function of the 5-HT7 receptor in the absence of the APP family members. Although also in this case compensatory effects might be troubling interpretation, it might allow us to discriminate between subtle differences in interactions between the different 'APP proteins'.

Alternatively, we recently acquired a rat neuroblastoma cell line, B103 (gift from Richard Dargusch,The Salk Institute, USA), which does not express APP nor APLP1/APLP2. Initial experiments are ongoing in this cell line to study whether the absence of endogenous levels of the 'APP family of proteins' alters the transport and/or localization of the 5-HT7 receptor and whether this has functional consequences as to 5-HT7 receptor mediated signalling (increase in cAMP, Erk1/2 activation, desensitisation).

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Coding of action categories in primate cortex.

We have continued a single cell study on the coding by macaque temporal cortical neurons of dynamic images of actions using a parameterized stimulus set and also studied the effect of categorization learning on the selectivity of macaque inferior temporal (IT) cortical neurons using parameterized sets of static shapes.

1. Coding of macaque temporal neurons to dynamic action stimuli.

The recognition of biological movements is extremely important for reproductive success and survival. Indeed, proper action recognition is essential for normal sexual and social behavior. Single cell work in non human primates as well as lesion studies and functional imaging in humans have suggested that the Superior Temporal Sulcus (STS) in the temporal lobe, the parietal cortex, and frontal areas are important for action recognition. The present study aims at a detailed understanding of the coding of dynamic images of actions in the temporal cortex. As a first step, we studied the responses of macaque temporal cortical neurons to a parameterized set of dynamic visual images of actions while the animals (two monkey subjects) were passively fixating and were not required to categorize the different actions. The parameterized stimulus set was developed in collaboration with Dr. F. Pollick (Univ. Glasgow, UK). The use of a fixation task instead of a discrimination or categorization task enabled us to test the neurons using a relatively wide variety of images and control stimuli, which is difficult in animals performing a discrimination of the stimuli.

We have examined the coding of a parameterized set of dynamic images of simple visual actions by macaque inferior temporal neurons. We used simple arm actions like knocking, lifting and throwing. The action images were rendered as stick figures. The objects of the goal-directed actions were not rendered. The experimental design that we employed to test the responses of visual neurons had several novel features. Firstly, we used a parameterized set of actions that consisted of three-way blends of 3 prototypical arm actions. The blending algorithm provided actions that consisted of mixtures of the three prototypes using different weights (20% steps) of each prototype (e.g. 0% throw, 80% lift, 20% knock; 20% throw, 60% lift, 20% knock, etc). This parameterized set of actions can be represented as a triangle with the prototypical actions at its corners and the different blends in between. The blending operation results in smooth transitions between the different action stimuli and allows a measurement of the tuning to the action stimuli in this action space.

Secondly, we systematically compared the responses of the neurons to the dynamic action stimuli to static snapshots of the action sequences so that we could assess the contribution of form versus motion information to the responses of the full actions. In addition, we measured the neuronal responses to snapshots translating in two opposite directions, to assess direction and motion selectivity. Thirdly, since the actions were restricted to one limb we could systematically reduce the full (stick-) body displays to more simple displays consisting of e.g. the moving limb only or even the end-effector dot only. This enabled us to determine the contribution of the body (stick-) configuration to the responses of the neurons to the full action stimuli. Additional tests included

presentations of the actions at different spatial positions, speeds and a reversal of the action sequences. The animals performed a fixation task during stimulus presentation and were not required to categorize or discriminate the action stimuli. We have recorded mainly in the dorsal and ventral bank of the rostral Superior Temporal Sulcus (rostral STS; visual areas STP and TE), but some of the recordings also included the lateral convexity of the inferior temporal cortex.

Preliminary analysis of the results of recordings in two animals (n= 217 neurons) indicates that rostral STS neurons respond selectively to temporal segments of the action sequences, but not to the whole action as such. Nonetheless, as a population, the neurons represented the similarity among the different actions. This was shown by performing a non-linear multidimensional scaling (ISOMAP) of the pairwise differences between the neural responses to the different stimuli: the low-dimensional ISOMAP configuration based on the neural responses corresponded at the ordinal level to that of the parametric configuration. Thus, as we have shown before for static shapes (Op de Beeck et al., Nature Neurosci., 2001), temporal cortical neurons can represent the similarity between dynamic action stimuli. Such representation of the similarity of action stimuli can be used as input for an action categorization process. Interestingly, the average population responses were significantly larger for the prototypical actions than for the blends. This bias for the extremities of the parametric configuration has also been observed for static shapes (Kayaert et al., Eur. J Neurosci., 2005) and face stimuli in IT (Leopold et al., Nature, 2006).

Based on our different tests, we could distinguish different kinds of neuronal selectivities. Firstly, neurons, mainly in the ventral bank of the rostral STS and the lateral convexity, responded as well to the action stimuli as to presentations of static snapshots and responded less to the moving arm alone than to whole body configuration. These neurons clearly responded to form information. These "snapshot" neurons can contribute to action coding by analyzing the form of the actor (Lange et al., J. Vision, 2006; Giese and Poggio, Nature Reviews: Neurosci., 2003). Secondly, other neurons, mainly in the ventral bank of the rostral STS, responded also equally well to the dynamic images as to the static snapshots, but responded as well to the moving arm alone as to the whole body configuration. A third category of neurons, mainly in the dorsal bank of the rostral STS responded much less to static snapshots than to the dynamic action images, clearly responding to motion information. Most of these neurons responded as well to the whole body configuration as to the moving arm alone. In fact, further testing showed that most of these neurons responded at least equally well to the motion of the hand dot alone. These strong selective responses to the motion of the end-effector – the wrist point – were surprising at first. However, selective responses to the end-effector are compatible with action coding since most of the information about the action is present precisely in the motion pattern of the end-effector. Part of these results were presented at the meeting of the Society for Neuroscience (Atlanta, October, 2006) and a preliminary version of the results appeared in abstract form. We are close to finishing the data collection and will analyze in depth the single cell data of the two animals.

2. Effects of categorization learning on selectivity of macaque inferior temporal neurons using static shapes.

Several studies, starting with Vogels (Europ. J. Neurosci., 1999), suggest that IT neurons do not represent categories of static images as such but instead show selectivity for exemplars of the same category (within-category selectivity) as well as for exemplars of different categories (between-category selectivity). Still, it is possible that the tuning of IT neurons is influenced by categorization learning. Indeed, it has been suggested that the responses of IT neurons reflect the perceptual similarity among stimuli (Kayaert et al., J. Neurosci.,2003; Op de Beeck et al., Nature Neurosci., 2001; see above) and the latter could be affected by categorization: stimuli are perceptually more distinct along stimulus dimensions relevant for categorization than along dimensions irrelevant for categorization. Thus it is possible that IT neurons are more narrowly tuned along categorization-relevant stimulus dimensions than along irrelevant dimensions or that IT neurons differentiate better exemplars belonging to different learned categories than exemplars belonging to the same categories. Previous studies have reported diverse effects of the categorization of images on the selectivity of neurons in macaque inferior temporal cortex for these images. Cells in IT cortex showed very weak category effects (Freedman et al., J. Neurosci., 2003): responses to shapes that belong to the same learned category were slightly more similar than responses to shapes that belong to different learned categories. However, this small effect could have been due to physical differences amongst the stimuli instead of differences in category membership. Another study reported that the IT representation of features that were diagnostic for distinguishing between two learned categories of stimuli was enhanced compared to the representation of non-diagnostic features (Sigala et al., Nature, 2002). However, this relatively large effect of categorization relevant feature selectivity can be due to mere stimulus selectivity, unrelated to categorization learning, since no pre-training measurements of selectivity were obtained and the diagnostic features were identical in both animals. Neither such expansion of the relevant dimension, nor any other metric change, was induced in a study where different categorization rules based on integral dimensions were applied (Op de Beeck et al., Nature Neurosci., 2001).

In the present study we controlled for prelearned stimulus selectivity effects versus learned category-related effects in two ways. First, we recorded the selectivity of IT neurons before and after categorization learning, and, the relevant categorization dimensions were counterbalanced across animals. In our study, 4 sets of 2D shapes were created combining the shape dimensions "aspect ratio" and "curvature", which were separable as tested beforehand in humans. A psychophysical study in humans (Ons et al., ECVP, 2006) showed that learning to categorize these shapes resulted in an improved discriminability for the relevant shape dimension. In a first part of our experiment, we recorded the responses of single IT neurons while presenting all shapes randomly intermixed for 100ms/image in a Rapid Serial Visual Presentation paradigm while the monkeys were passively fixating. Afterwards, we trained these monkeys to group stimuli into 2 categories with curvature and aspect ratio as the relevant dimension for the first and second monkey, respectively. After training, we recorded the responses of single IT neurons to the shapes of the 4 sets while the monkeys were categorizing the images.

Our preliminary results suggest that learning to categorize stimuli can alter the selectivity of IT neurons: training shifted the ratio between within-category response similarities and between-category response similarities to a larger value for the relevant dimension. However, this effect was small, but significant. We found no clear evidence for an expansion of the relevant dimension or contraction of the irrelevant dimension. These results suggest that shape categorization learning can induce minor changes in the shape tuning of IT neurons. It is possible that the categorization learning may also have affected other measures of the selective responses of IT neurons that were not looked at in previous studies. This is currently under investigation.

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