J Neural Transm (2008) 115:1165–1172
DOI 10.1007/s00702-008-0067-y

ALZHEIMER'S DISEASE AND RELATED DISORDERS - ORIGINAL ARTICLE

Selective loss of $P2Y_2$ nucleotide receptor immunoreactivity is associated with Alzheimer's disease neuropathology

Mitchell K. P. Lai · Michelle G. K. Tan · Sara Kirvell · Carl Hobbs · Jasinda Lee · Margaret M. Esiri · Christopher P. Chen · Paul T. Francis

Received: 19 March 2008 / Accepted: 5 May 2008 / Published online: 28 May 2008
© Springer-Verlag 2008

Abstract The uridine nucleotide-activated $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors are widely expressed in the brain and are involved in many CNS processes, including those which malfunction in Alzheimer's disease (AD). However, the status of these receptors in the AD neocortex, as well as their putative roles in the pathogenesis of neuritic plaques and neurofibrillary tangles, remain unclear. In this study, we used immunoblotting to measure $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors in two regions of the postmortem neocortex of neuropathologically assessed AD patients and aged controls. $P2Y_2$ immunoreactivity was found to be selectively reduced in the AD parietal cortex, while $P2Y_4$ and $P2Y_6$ levels were unchanged. In contrast, all three receptors were preserved in the occipital cortex, which is known to be minimally affected by AD neuropathology. Furthermore, reductions in parietal $P2Y_2$ immunoreactivity correlated both with neuropathologic scores and markers of synapse loss. These results provide a basis for considering $P2Y_2$ receptor changes as a neurochemical substrate of AD, and point towards uridine nucleotide-activated P2Y receptors as novel targets for disease-modifying AD pharmacotherapeutic strategies.

Keywords Alzheimer's disease · Uridine nucleotide · Purinergic P2Y receptors · Amyloid plaque · Neurofibrillary tangle · Neocortex

M. K. P. Lai (✉) · M. G. K. Tan
Dementia Research Laboratory, Department of Clinical Research, Singapore General Hospital, Outram Road, Singapore 169608, Singapore
e-mail: mitchell.lai@dementia-research.org

M. K. P. Lai · J. Lee · C. P. Chen
Department of Pharmacology, Yong Loo Lin School of Medicine, National University of Singapore, Kent Ridge, Singapore 117597, Singapore

S. Kirvell · C. Hobbs · P. T. Francis
Wolfson Centre for Age-Related Diseases, King's College London, London SE1 1UL, UK

M. M. Esiri
Department of Neuropathology, John Radcliffe Hospital, University of Oxford, Oxford OX3 9DS, UK

Introduction

The neuropathologic hallmarks of Alzheimer's disease (AD) include β-amyloid (Aβ) containing neuritic plaques, neurofibrillary tangles arising from paired helical filaments of hyperphosphorylated τ proteins, and neuronal degeneration across multiple transmitter systems. In particular, losses of cholinergic as well as serotonergic neurons are thought to underlie much of the neurochemical perturbations found in the AD cortex and contribute to the development of cognitive and behavioral symptoms (Cummings and Back 1998; Francis et al. 1999; Lanari et al. 2006; Lanctot et al. 2001). Dysfunction of acetylcholine and serotonin G-protein coupled receptors, especially those which associate with $G\alpha_{q/11}$-subtypes which in turn activate phopholipase C, calcium mobilization and protein kinase C, has been reported in AD. For example, muscarinic M1 receptors are found to be uncoupled to their G-proteins (Flynn et al. 1991; Tsang et al. 2006), while specific reductions of serotonin 5-HT_{2A} receptors occur in the neocortex (Cross et al. 1986; Lai et al. 2005). Given their roles in emotive and behavioral processes, memory and cognition, as well as in intracellular signaling events which regulates amyloidogenic processing of amyloid precursor protein (APP) and τ phosphorylation, neurochemical perturbations of $G\alpha_{q/11}$-coupled receptors may be involved in both clinical and neuropathologic

features of AD (Hellstrom-Lindahl 2000; Lai et al. 2005; Meneses and Hong 1997; Nitsch et al. 1992, 1996; Tsang et al. 2006, 2007). Besides cholinergic, serotonergic and other well studied neurotransmitter systems, there has been growing awareness of the neuropsychiatric and neurobiological relevance of various "non-classical" neurotransmitters like neuropeptides, nitric oxide and nucleotides, with their status in AD brain only just beginning to be investigated. For example, the large P2Y family of receptors which are activated by purine or pyrimidine nucleotides, are known to be expressed in diverse tissue types in humans and subserve multiple physiological functions (Abbracchio et al. 2006). Of these, the $P2Y_2$ receptors are responsive to both ATP and UTP, while $P2Y_4$ receptors recognize UTP, and $P2Y_6$ is UDP-selective (Nicholas et al. 1996). $P2Y_{2,4,6}$ receptors have been detected in both neuronal and glial cells in the CNS (reviewed in Hussl and Boehm 2006), where they mediate a variety of processes of potential relevance to AD. For example, $P2Y_2$ receptor activation enhances the proteolytic processing of APP in a manner which precludes the formation of Aβ, the major constituent of neuritic plaques (Camden et al. 2005). $P2Y_2$ receptors also mediate neuronal differentiation via nerve growth factor/TrkA signaling (Arthur et al. 2005), while $P2Y_{2,4,6}$ receptors have been reported to have neuroprotective, antiapoptotic or proapoptotic functions, as well as the ability to modulate glutamate *N*-methyl-D-aspartate (NMDA) receptor currents or intracellular calcium (Calvert et al. 2004; Cavaliere et al. 2004, 2005; Kim et al. 2003; Lee et al. 2007; Wirkner et al. 2007). Given that the AD brain is characterized by formation and aggregation of Aβ, loss of trophic support, cell death and apoptosis, derangement of intracellular calcium-mediated signaling pathways, as well as glutamatergic deficits (Behl 2000; Cole et al. 1988; Counts and Mufson 2005; Francis 2003; Selkoe 2001; Tsang et al. 2007), it is reasonable to hypothesize that $P2Y_{2,4,6}$ receptor dysfunction may be involved in the AD process. However, the status of $P2Y_{2,4,6}$ receptors in the AD brain is at present unclear. Here, using postmortem materials from a cohort of longitudinally assessed AD patients as well as matched controls, we measured $P2Y_{2,4,6}$ receptors in two brain regions known to be differentially affected by AD neuropathology (parietal and occipital cortex) and investigated the associations between receptor immunoreactivities and neuropathologic data.

Materials and methods

Patients, clinical and neuropathologic assessments

The study comprised of up to 29 AD patients and 12 elderly controls. The AD patients were derived from a cohort of community-living, clinically diagnosed dementia patients recruited into a longitudinal study of behavior in dementia based in Oxfordshire, UK (Hope et al. 1999). The inclusion and exclusion criteria, point of study entry characteristics, as well as natural history of the study cohort have previously been described in detail (Hope et al. 1997a, b, 1999). From study entry till death, cognitive functioning of the patients was assessed every 4 months with the mini-mental state examination (Folstein et al. 1975). The mean of up to five MMSE scores before death (MMSE5) was used as an indicator of dementia severity to avoid floor effects (Lai et al. 2001). Drug histories were recorded for all patients: eight were on neuroleptics, seven were on minor tranquilizers, and only two were given tricyclic antidepressants in the 8 months before death. No patient was on cholinergic replacement therapies. At death, informed consent was obtained from the patients' next-of-kin for the removal of brain, and selection of subjects for this study is based on the CERAD neuropathologic diagnosis of AD (Mirra et al. 1991) as well as tissue availability. Only one patient in this study had evidence of mixed AD and vascular dementia. As an additional indicator of disease severity, paraffin-embedded blocks of temporal cortex were stained with methenamine silver and a modified Palmgren stain for a blinded, semi-quantitative rating (from 0, absent to 3, highest) of neuritic plaques (NP) and neurofibrillary tangles (NFT) as previously described (Jacobs et al. 2006). Patients with scores of 0–2 for NP or NFT were categorized under the 'Low/Moderate' NP or NFT rating group; while scores of 3 were in the 'High' group. The age-matched controls did not have dementia or other neurological diseases.

Tissue processing and brain membrane homogenate preparation

Gray matter from the angular gyrus in the parietal lobe (Brodmann Area, BA 39) and the primary visual cortex in the occipital lobe (BA 17) was homogenized and processed for immunoblotting as previously described with slight modifications (Kirvell et al. 2006). In brief, after dissection to remove meninges and white matter, aliquots of tissue was homogenized in ice-cold 50 mm Tris–HCl buffer (pH 7.4, with 5 mm EGTA, 10 mm EDTA, 2 µg/ml aprotonin, 1 µg/ml E64, 2 µg/ml leupeptin hemisulphate, 2 µg/ml pepstatin A and 20 µg/ml phenylmethylsulphonyl fluoride) using a Teflon–glass homogeniser, followed by storage at −70°C. Protein concentration was measured with the Coomassie Plus reagent (Pierce Biotechnology Inc., Rockford, IL). pH was also measured in the cerebellum as an indicator of agonal status (Hardy et al. 1985). When ready for immunoblotting experiments, brain homogenates were thawed and mixed with Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA) 1:1 v/v followed by

boiling. Samples were not available from both regions for all cases.

Immunoblotting and Immunohistochemistry

Primary antibodies used in this study were anti-$P2Y_2$, anti-$P2Y_4$ and anti-$P2Y_6$ (rabbit polyclonals from Alomone Labs Ltd, Jerusalem, Israel) and anti-β-actin (mouse monoclonal from Sigma-Aldrich Ltd.). Boiled brain homogenate were electrophoretically separated on 10% polyacrylamide gels, transferred onto nitrocellulose membranes, and blocked in 10 mM phosphate buffered saline, pH 7.4, 0.1% Tween 20/5% skim milk (PBSTM) before immunoblotting with primary antibody at the following dilutions in PBSTM: 1:200 (anti-$P2Y_2$ and anti-$P2Y_6$), 1:300 (anti-$P2Y_4$), for 3 h at room temperature (RT) or overnight at 4°C. To assess specificity of antibody binding, certain membranes were pre-incubated with control peptide antigens for 1 h before incubation with primary antibodies. Following washings in PBSTM and incubation with horseradish peroxidase conjugated secondary antibodies (1:10,000, Jackson ImmunoResearch Inc., West Grove, PA), immunoreactive bands on the membranes were detected by enhanced chemiluminescence and quantified by an image analyzer (UVItec, UK). Membranes were then stripped and reblotted with anti-β-actin (1:5,000) to control for sample loading across lanes. One lane of external standard consisting of fixed amounts of protein from one homogenate sample was loaded for each membrane for normalization of data. Normalized immunoblot optical densities are expressed in arbitrary units. Immunoblotting data of anti-synaptophysin (1:20,000, Sigma Chemical Co., Poole, UK) using unboiled aliquots of the same homogenates as the P2Y studies have been previously reported (Kirvell et al. 2006). For immunohistochemical detection of $P2Y_2$ staining, 10 μm paraffin wax sections of rat prefrontal cortex were dewaxed in xylene for 10 min followed by washes through a descending series of alcohols (100–50%) into water. Sections were then immersed in boiling 10 mM citric acid (pH 6) before being microwave pressure cooked for a further 10 min in the same buffer, followed by treatment in 0.3% H_2O_2 (v/v) in phosphate buffered saline (PBS) for 30 min, RT. Then, the sections were washed in PBS, pre-blocked in 1.5% normal goat serum (v/v)/0.1% bovine serum albumin (BSA) (w/v) in PBS, RT before overnight incubation at 4°C with anti-$P2Y_2$ (1:200) diluted in 0.1% BSA/PBS. On the following day, sections were washed in PBS and further incubated in biotinylated goat anti-rabbit IgG (1:400, Vector labs, USA) for 1 h, RT. Sections were subsequently incubated in avidin–biotin complex (Vector Labs, USA) for 1 h prior to visualization using 0.06% DAB (3,3′-diaminobenzidine) (w/v) prepared in PBS together with 0.01% H_2O_2 (v/v). Sections were then thoroughly washed in running tap water and counterstained in hematoxylin before being dehydrated through an ascending series of alcohols (50–100%) into xylene and coverslips applied with DPX (dibutyl phthalate xylene) mountant. Slides were then viewed using a Carl Zeiss Axioskop microscope with JVC 3-CCD color camera model KY-F55B and images captured using Carl Zeiss KS300 Image Analysis Software Version 3.

Statistical analyses

Data were first tested for normality for the selection of parametric or non-parametric tests. Potential confounding effects of demographic or disease variables on P2Y receptor immunoblot data, as well as inter-correlations among variables, were studied by Pearson's product moment or Spearman's correlation. Demographic variables and P2Y receptor levels between controls and AD were compared with Student's t tests, while comparisons of immunoblot data among controls, AD with 'Low/Moderate' neuropathologic rating, as well as AD with 'High' rating, were performed using one-way ANOVA followed by post hoc Scheffé tests.

Results

Specificity of $P2Y_{2,4,6}$ receptor antibodies

The polyclonal antibodies to $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors produced major bands at approximately 60, 75 and 60 kDa, respectively, on immunoblotting with control and AD brain homogenates, as well as with neuroblastoma SH-SY5Y cell lysate (Fig. 1), consistent with the manufacturer's data sheets. Occasionally, there are additional, smaller molecular weight bands (30–50 kDa) detected by the $P2Y_2$ antibody in both AD and control homogenates which may represent degradation products or isoforms of the receptor. Except for a weak correlation of the 50 kDa minor bands in BA17 with cerebellum pH (Pearson $r = -0.42$, $p = 0.04$), these minor bands did not correlate with markers of disease and tissue quality (NP, NFT, postmortem interval). Such smaller bands were not routinely seen for $P2Y_4$ and $P2Y_6$ blots. Because we are currently unable to reliably identify the minor bands, analyses were restricted to the major 60 kDa band for $P2Y_2$ (see Fig. 2a). As shown in Fig. 1, the antibodies seemed to specifically detect their target receptors, since preincubation of the antibodies with peptides corresponding to unique sequences of the respective receptors (see Fig. 1 legend) inhibited much of the signal seen in parallel immunoblots without peptide preincubation.

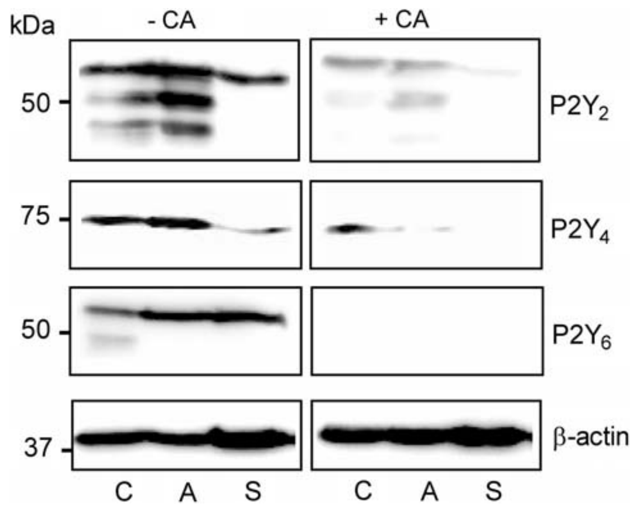

Fig. 1 Parallel lanes of brain homogenates from *C* control, *A* AD and *S* SH-SY5Y lysate were immunoblotted with anti-$P2Y_2$, anti-$P2Y_4$ and anti-$P2Y_6$, with (+*CA*) or without (−*CA*) preincubation with equimolar control antigen peptides ($P2Y_2$ residues 227–244: KPAYGTTGL-PRAKRKSVR; $P2Y_4$ residues 337–350: HEESISRWADTHQD; $P2Y_6$ residues 311–328: QPHDLLQKLTAKWQRQRV). The −CA and +CA blots were run, blotted and visualized using enhanced chemiluminescence in parallel

Demographic, disease and $P2Y_{2,4,6}$ immunoblot variables in control and AD

Demographic variables were matched between the AD subjects and controls (Student's $p > 0.05$) except for lower pH in AD (Table 1), suggesting more severe brain acidosis in AD from prolonged agonal state (Hardy et al. 1985). However, pH, as well as other demographic variables listed in Table 1, did not correlate with $P2Y_{2,4,6}$ immunoblot densities (Pearson $p > 0.05$). As expected, both NP and NFT scores were significantly higher in AD compared to controls (Table 1). NP and NFT scores were also highly correlated with each other (Spearman $\rho = 0.915, p < 0.001$), suggesting an absence of 'plaque predominant' or 'tangle predominant' neuropathology in this cohort. In the occipital cortex (BA17), $P2Y_2$, $P2Y_4$, and $P2Y_6$ immunoblot densities were not significantly different between controls and AD, while in the parietal cortex (BA39), $P2Y_2$ levels were significantly reduced in AD, with no change in $P2Y_4$, and $P2Y_6$ densities (Fig. 2b). Lastly, immunoblot densities did not differ significantly between patients who were administered neuroleptic or minor tranquilizers in the 8 months before death and those not on such medication (data not shown).

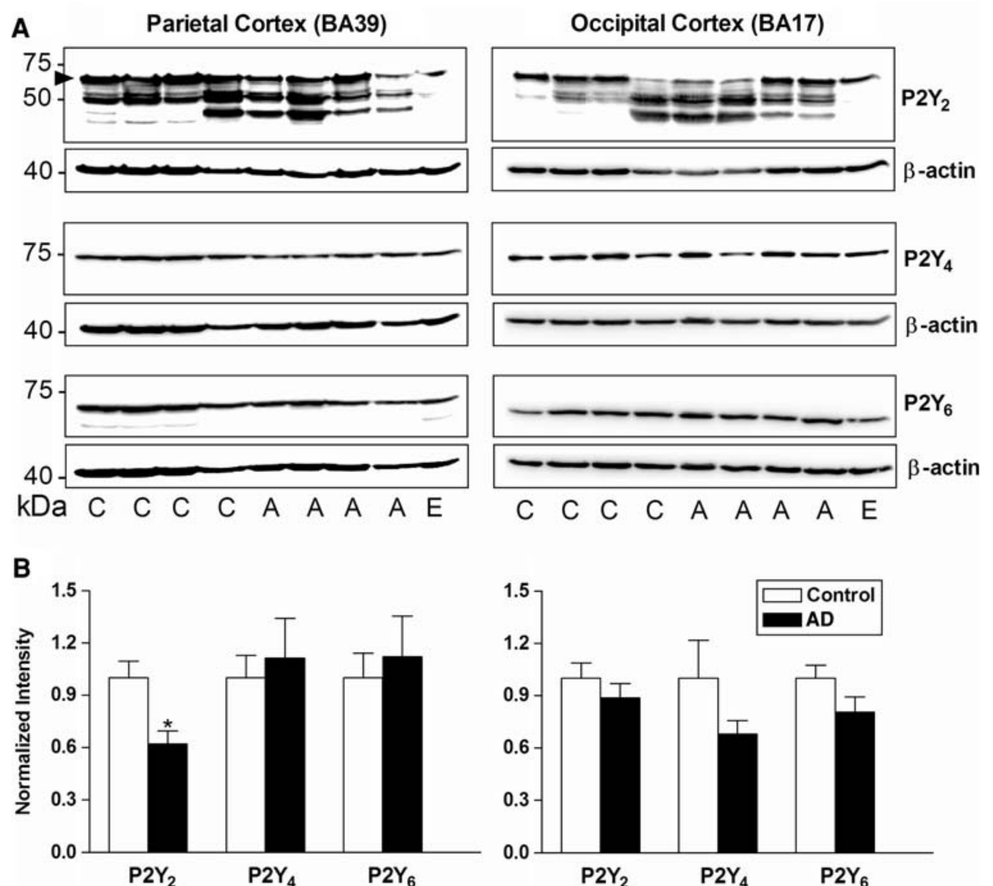

Fig. 2 **a** Representative blots of immunoreactivity to $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors in control (n = 9–10) and AD (n = 21–22) homogenates from the parietal and occipital cortex. Membranes were stripped and reblotted with anti-β-actin for normalization of protein loading. *Lanes* are *C* control, *A* AD, and *E* external standard. Indicative positions of molecular weight markers are in kilodaltons (kDa). For $P2Y_2$, only the 60 kDa bands (indicated by *arrow head*) were analyzed (see text). **b** Bar graphs of mean immunoblot densities ± SEM (in arbitrary units). *Significantly different from controls. Student's t test, p = 0.009

Table 1 Demographic and disease variables in a cohort of prospectively assessed AD patients and controls

	AD	Control
Maximum number of cases[a]	29	12
Age at death (years)	81.9 ± 1	77.2 ± 3
Sex (M/F)	16/13	6/6
Post-mortem interval (h)	40.5 ± 5	39.3 ± 5
pH[b]	6.31 ± 0.07*	6.61 ± 0.1
NFT[c]	2.28 ± 0.18**	0.09 ± 0.09
NP[c]	2.28 ± 0.16**	0.18 ± 0.1
Disease duration (years)	9.6 ± 0.9	NA
MMSE5	5.65 ± 1.28	NA

Data are mean ± SEM

MMSE5 mean of ≤5 mini-mental state examination scores predeath (see text), *NA* not applicable, *NFT* neurofibrillary tangle score, *NP* neuritic plaque score

* Different from control $p < 0.05$

** Different from control $p < 0.001$

[a] Not all neurochemical measures were available for all cases. The *N* values available for each region or score are listed in the figure legends

[b] Measured in the cerebellum as an indicator of agonal status (Hardy et al. 1985)

[c] Data for NFT and NP scores were available for 11 controls and 25 AD patients

Association of $P2Y_{2,4,6}$ immunoblot variables with neuropathologic and clinical features in AD

Of the receptors studied, only $P2Y_2$ density in BA39 inversely correlated with NP (Spearman $\rho = -0.69$, $p < 0.01$) and NFT (Spearman $\rho = -0.67$, $p < 0.01$) ratings. $P2Y_4$ and $P2Y_6$ in BA39 did not correlate with neuropathologic scores, and all receptors in both regions did not correlate with disease duration or with dementia severity as indicated by MMSE5 (Spearman $p > 0.05$). Furthermore, among controls and neuropathologic subgroups of AD, $P2Y_2$ densities in BA39 were significantly reduced only in patients in the 'High' rating group for NP and NFT (Fig. 3a). Lastly, $P2Y_2$ densities in BA39 significantly correlated with previously reported synaptophysin immunoreactivity in BA39 in the subset of patients for whom both measures were available (Fig. 3b). Synaptophysin levels did not correlate with $P2Y_4$ or $P2Y_6$ densities (Pearson $p > 0.05$).

Discussion

At present, one potential pharmacotherapeutic utility of P2Y receptor antagonists in AD has been proposed based on their efficacy in limiting ATP-mediated chronic inflammation responses as well as excitotoxicity arising from excessive activation of glutamate receptors (reviewed in Burnstock 2007). However, apart from $P2Y_1$ receptors which have been immunohistochemically localized to neuritic plaques and neurofibrillary tangles (Moore et al. 2000), it is unclear whether other $G\alpha_{q/11}$-coupled P2Y receptors are altered in the AD neocortex, and whether these alterations are associated with clinical or neuropathologic features of AD. Here, we report the status of $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors in the AD neocortex as well as their correlations with dementia severity, neuritic plaque and neurofibrillary tangle burden. Interestingly, P2Y receptor deficits seem to be subtype-specific, as we found reductions of $P2Y_2$ while $P2Y_4$ and $P2Y_6$ receptors remained unchanged. Additionally, $P2Y_2$ deficits seemed to be associated with plaque and tangle burden, since the loss of $P2Y_2$ immunoreactivity was restricted to the parietal cortex, whereas levels were preserved in the occipital cortex, a region considered to be minimally affected by AD

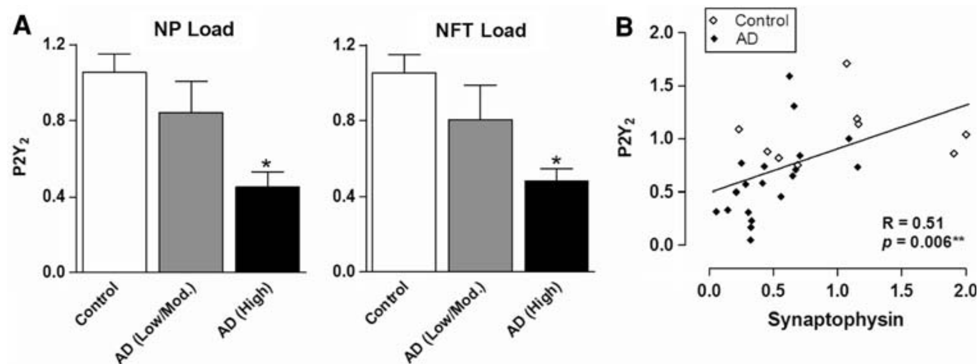

Fig. 3 **a** Bar graphs of mean ± SEM normalized $P2Y_2$ receptor immunoreactivity (in arbitrary units) in BA39 across controls ($n = 9$) and AD with 'Low/Moderate' ($n = 8$) or 'High' ($n = 9$) neuritic plaque (*NP*) and neurofibrillary tangle (*NFT*) ratings. **b** Scatter plot of normalized $P2Y_2$ immunoreactivity with synaptophysin immunoreactivity in BA39 of available subjects (9 controls, 19 AD), both in arbitrary units. *Significantly different from control (one-way ANOVA with post hoc Scheffé $p < 0.01$). **Significant Pearson correlation

neuropathology even in advanced stages of disease (Braak and Braak 1991; Lerch et al. 2005). Furthermore, when AD patients were grouped according to plaque and tangle severity, parietal $P2Y_2$ loss was significant only in subgroups with 'High' ratings for NP and NFT, while patients labeled 'Low/Moderate' for NP and NFT had intermediate densities of $P2Y_2$ (Fig. 3a). Moreover, decreases in parietal $P2Y_2$ densities also correlated with lower synaptophysin immunoreactivity, a marker of synapse loss (Butler et al. 2007). Additionally, immunohistochemical staining in rat prefrontal cortex suggests that at least some of the $P2Y_2$-expressing cell bodies and processes are derived from pyramidal neurons (Fig. 4), which are known to play important roles in cognitive processes and are vulnerable to neurodegeneration (Francis et al. 1993; Mann 1996; Pearson and Powell 1989). With the putative demonstration of $P2Y_2$ localization in the cortex of normal rodents, it will be of interest to extend the study of the purinergic system to AD animal models in order to determine whether purinergic deficits result from pathophysiological processes which simulate AD, or whether manipulations of purinergic function would affect disease progression in the animal model.

In contrast to NP and NFT scores, $P2Y_{2,4,6}$ receptor immunoreactivity did not correlate with dementia severity, suggesting these receptors may play an indirect regulatory, rather than effector, role in processes mediating cognition and memory. For example, $P2Y_2$ receptor activation has been shown to regulate the expression of acetylcholinesterase and acetylcholine receptors in the neuromuscular junction (Tung et al. 2004), although further studies are needed to determine if $P2Y_2$ has similar effects on neurons. Taken together, these findings suggest a specific association between changes in $P2Y_2$ receptor immunoreactivity and neuropathologic features in the AD parietal cortex.

What are the likely mechanisms underlying the effect of $P2Y_2$ receptor loss on AD neuropathology? As $P2Y_2$ activation has been reported to regulate proteolytic processing of APP toward the non-amyloidogenic pathway (Camden et al. 2005), a loss of $P2Y_2$ may expedite the deposition of Aβ and the resultant aggregation of fibrillar Aβ and the formation of plaques, in a manner analogous to that proposed for cholinergic receptor deficits (Auld et al. 2002; Hellstrom-Lindahl 2000). Similarly, although there is as yet no evidence that $P2Y_2$ receptors regulate the phosphorylation of τ or other microtubule-associated proteins, it is conceivable that $G\alpha_{q/11}$-mediated signaling arising from $P2Y_2$ activation may lead to decreased τ phosphorylation, as is shown for $G\alpha_{q/11}$-coupled muscarinic receptors (Hellstrom-Lindahl 2000; Sadot et al. 1996). Furthermore, the correlation of $P2Y_2$ deficit with synapse loss (as indicated by reduced synaptophysin immunoreactivity) suggest a dysfunction of the trophic support which may normally be mediated partly by $P2Y_2$ receptors (Arthur et al. 2005; Gonzalez et al. 2005). Confirmation or refutation of these hypotheses will await further in vitro studies, but several limitations of the current study as well as further questions which arise need to be considered. Firstly, findings from the present correlational study cannot unequivocally demonstrate the role of $P2Y_2$ changes in plaque and tangle pathogenesis, since it is also possible that $P2Y_2$ receptor losses are a reflection of degenerating $P2Y_2$-expressing cells after exposure to Aβ. For example, perturbations of ATP induced Ca^{2+} fluxes have been reported in Aβ-treated fetal microglia in vitro (McLarnon et al. 2005). However, the scenario of Aβ-induced $P2Y_2$ deficit does not necessarily negate a potential role of $P2Y_2$ deficit (which resulted from Aβ neurotoxicity) in further exacerbating amyloidogenic processing. Indeed, the hypothesis that Aβ and receptor dysfunction interact in a vicious cycle leading to widespread neurodegeneration deserves further study. Furthermore, unlike cholinergic deficits which can be traced to degeneration of acetylcholine producing neurons in the basal forebrain (Whitehouse et al. 1982), the pathogenesis of reduced $P2Y_2$ immunoreactivity is unclear. It is also not known whether the selective reduction of $P2Y_2$ receptors in AD parietal cortex is due to selective vulnerability of populations of $P2Y_2$-bearing cells, or to specific downregulation or desensitization of $P2Y_2$ receptors. Lastly, although the correlation of $P2Y_2$ with

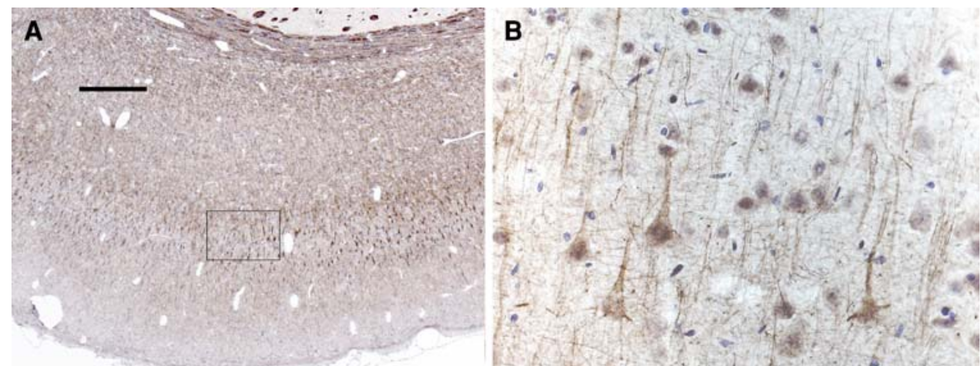

Fig. 4 **a** Immunohistochemistry of anti-$P2Y_2$ in rat prefrontal cortex (4× magnification), the *scale bar* indicating 50 µm. **b** 40× Magnification of area indicated by the *rectangle* from **a**, *arrows* indicating some of the pyramidal neurons which were positive for $P2Y_2$ immunoreactivity

synaptophysin immunoreactivity suggests perturbations in at least a proportion of neuronal $P2Y_2$ receptors, we are unable to conclude in the current study whether, or to what extent, glial $P2Y_2$ receptors are affected. Further studies employing quantitative immunohistochemical methods may be needed to address these questions.

In conclusion, in this first study to investigate the status of uridine nucleotide-activated $P2Y_{2,4,6}$ receptors in the postmortem neocortex of a cohort of well-characterized AD patients, we found selective reductions of $P2Y_2$ immunoreactivity in the parietal, but not occipital, cortex. We further showed that the loss of parietal $P2Y_2$ immunoreactivity correlated with neuritic plaque and neurofibrillary tangle burden. Although future studies are needed to further characterize the $P2Y_2$ changes, such as the cells types involved, the present results indicate a specific association between $P2Y_2$ receptor perturbations and neuropathologic features in AD. Our study suggests that the possible roles of $P2Y_2$ receptor alterations in regulating molecular processes underlying plaque and tangle formation should be further investigated using various approaches, including cell function assays, animal models and postmortem studies. Furthermore, P2Y ligands may warrant study as potential candidates for AD pharmacotherapy.

Acknowledgments This study is supported by the Wellcome Trust, UK. We thank Dr. Brendan McDonald for the collection and classification of the postmortem samples. S. Kirvell is the Edmond J. Safra Fellow at Wolfson CARD, King's College London. In Singapore, the work was supported by the National Medical Research Council (NMRC/0932/2005) to M. K. P. Lai and a Faculty Start-up grant to C. P. Chen. M. K. P. Lai would like to thank Olivia H. J. Oon for valuable assistance.

References

Abbracchio MP, Burnstock G, Boeynaems JM, Barnard EA, Boyer JL, Kennedy C, Knight GE, Fumagalli M, Gachet C, Jacobson KA, Weisman GA (2006) International Union of Pharmacology LVIII: update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy. Pharmacol Rev 58:281–341

Arthur DB, Akassoglou K, Insel PA (2005) $P2Y_2$ receptor activates nerve growth factor/TrkA signaling to enhance neuronal differentiation. Proc Natl Acad Sci USA 102:19138–19143

Auld DS, Kornecook TJ, Bastianetto S, Quirion R (2002) Alzheimer's disease and the basal forebrain cholinergic system: relations to β-amyloid peptides, cognition, and treatment strategies. Prog Neurobiol 68:209–245

Behl C (2000) Apoptosis and Alzheimer's disease. J Neural Transm 107:1325–1344

Braak H, Braak E (1991) Neuropathological stageing of Alzheimer-related changes. Acta Neuropathol (Berl) 82:239–259

Burnstock G (2007) Physiology and pathophysiology of purinergic neurotransmission. Physiol Rev 87:659–797

Butler D, Bendiske J, Michaelis ML, Karanian DA, Bahr BA (2007) Microtubule-stabilizing agent prevents protein accumulation-induced loss of synaptic markers. Eur J Pharmacol 562:20–27

Calvert JA, Atterbury-Thomas AE, Leon C, Forsythe ID, Gachet C, Evans RJ (2004) Evidence for $P2Y_1$, $P2Y_2$, $P2Y_6$ and atypical UTP-sensitive receptors coupled to rises in intracellular calcium in mouse cultured superior cervical ganglion neurons and glia. Br J Pharmacol 143:525–532

Camden JM, Schrader AM, Camden RE, Gonzalez FA, Erb L, Seye CI, Weisman GA (2005) $P2Y_2$ nucleotide receptors enhance α-secretase-dependent amyloid precursor protein processing. J Biol Chem 280:18696–18702

Cavaliere F, Amadio S, Angelini DF, Sancesario G, Bernardi G, Volonte C (2004) Role of the metabotropic $P2Y_4$ receptor during hypoglycemia: cross talk with the ionotropic NMDAR1 receptor. Exp Cell Res 300:149–158

Cavaliere F, Nestola V, Amadio S, D'Ambrosi N, Angelini DF, Sancesario G, Bernardi G, Volonte C (2005) The metabotropic $P2Y_4$ receptor participates in the commitment to differentiation and cell death of human neuroblastoma SH-SY5Y cells. Neurobiol Dis 18:100–109

Cole G, Dobkins KR, Hansen LA, Terry RD, Saitoh T (1988) Decreased levels of protein kinase C in Alzheimer brain. Brain Res 452:165–174

Counts SE, Mufson EJ (2005) The role of nerve growth factor receptors in cholinergic basal forebrain degeneration in prodromal Alzheimer disease. J Neuropathol Exp Neurol 64:263–272

Cross AJ, Crow TJ, Ferrier IN, Johnson JA (1986) The selectivity of the reduction of serotonin S2 receptors in Alzheimer-type dementia. Neurobiol Aging 7:3–7

Cummings JL, Back C (1998) The cholinergic hypothesis of neuropsychiatric symptoms in Alzheimer's disease. Am J Geriatr Psychiatry 6:S64–S78

Flynn DD, Weinstein DA, Mash DC (1991) Loss of high-affinity agonist binding to M1 muscarinic receptors in Alzheimer's disease: implications for the failure of cholinergic replacement therapies. Ann Neurol 29:256–262

Folstein MF, Folstein SE, McHugh PR (1975) Mini-mental state. A practical method for grading the cognitive state of patients for the clinician. J Psychiatr Res 12:189–198

Francis PT (2003) Glutamatergic systems in Alzheimer's disease. Int J Geriatr Psychiatry 18:S15–S21

Francis PT, Sims NR, Procter AW, Bowen DM (1993) Cortical pyramidal neurone loss may cause glutamatergic hypoactivity and cognitive impairment in Alzheimer's disease: investigative and therapeutic perspectives. J Neurochem 60:1589–1604

Francis PT, Palmer AM, Snape M, Wilcock GK (1999) The cholinergic hypothesis of Alzheimer's disease: a review of progress. J Neurol Neurosurg Psychiatry 66:137–147

Gonzalez FA, Weisman GA, Erb L, Seye CI, Sun GY, Velazquez B, Hernandez-Perez M, Chorna NE (2005) Mechanisms for inhibition of P2 receptors signaling in neural cells. Mol Neurobiol 31:65–79

Hardy JA, Wester P, Winblad B, Gezelius C, Bring G, Eriksson A (1985) The patients dying after long terminal phase have acidotic brains; implications for biochemical measurements on autopsy tissue. J Neural Transm 61:253–264

Hellstrom-Lindahl E (2000) Modulation of β-amyloid precursor protein processing and τ phosphorylation by acetylcholine receptors. Eur J Pharmacol 393:255–263

Hope T, Keene J, Fairburn C, McShane R, Jacoby R (1997a) Behaviour changes in dementia. 2: Are there behavioural syndromes? Int J Geriatr Psychiatry 12:1074–1078

Hope T, Keene J, Gedling K, Cooper S, Fairburn C, Jacoby R (1997b) Behaviour changes in dementia. 1: Point of entry data of a prospective study. Int J Geriatr Psychiatry 12:1062–1073

Hope T, Keene J, Fairburn CG, Jacoby R, McShane R (1999) Natural history of behavioural changes and psychiatric symptoms in Alzheimer's disease. A longitudinal study. Br J Psychiatry 174:39–44

Hussl S, Boehm S (2006) Functions of neuronal P2Y receptors. Pflugers Arch 452:538–551

Jacobs EH, Williams RJ, Francis PT (2006) Cyclin-dependent kinase 5, Munc18a and Munc18-interacting protein 1/X11α protein up-regulation in Alzheimer's disease. Neuroscience 138:511–522

Kim SG, Soltysiak KA, Gao ZG, Chang TS, Chung E, Jacobson KA (2003) Tumor necrosis factor α-induced apoptosis in astrocytes is prevented by the activation of $P2Y_6$, but not $P2Y_4$ nucleotide receptors. Biochem Pharmacol 65:923–931

Kirvell SL, Esiri M, Francis PT (2006) Down-regulation of vesicular glutamate transporters precedes cell loss and pathology in Alzheimer's disease. J Neurochem 98:939–950

Lai MK, Lai OF, Keene J, Esiri MM, Francis PT, Hope T, Chen CP (2001) Psychosis of Alzheimer's disease is associated with elevated muscarinic M_2 binding in the cortex. Neurology 57:805–811

Lai MK, Tsang SW, Alder JT, Keene J, Hope T, Esiri MM, Francis PT, Chen CP (2005) Loss of serotonin 5-HT_{2A} receptors in the postmortem temporal cortex correlates with rate of cognitive decline in Alzheimer's disease. Psychopharmacology (Berl) 179:673–677

Lanari A, Amenta F, Silvestrelli G, Tomassoni D, Parnetti L (2006) Neurotransmitter deficits in behavioural and psychological symptoms of Alzheimer's disease. Mech Ageing Dev 127:158–165

Lanctot KL, Herrmann N, Mazzotta P (2001) Role of serotonin in the behavioral and psychological symptoms of dementia. J Neuropsychiatry Clin Neurosci 13:5–21

Lee CJ, Mannaioni G, Yuan H, Woo DH, Gingrich MB, Traynelis SF (2007) Astrocytic control of synaptic NMDA receptors. J Physiol 581:1057–1081

Lerch JP, Pruessner JC, Zijdenbos A, Hampel H, Teipel SJ, Evans AC (2005) Focal decline of cortical thickness in Alzheimer's disease identified by computational neuroanatomy. Cereb Cortex 15:995–1001

Mann DM (1996) Pyramidal nerve cell loss in Alzheimer's disease. Neurodegeneration 5:423–427

McLarnon JG, Choi HB, Lue LF, Walker DG, Kim SU (2005) Perturbations in calcium-mediated signal transduction in microglia from Alzheimer's disease patients. J Neurosci Res 81:426–435

Meneses A, Hong E (1997) Role of 5-HT_{1B}, 5-HT_{2A} and 5-HT_{2C} receptors in learning. Behav Brain Res 87:105–110

Mirra SS, Heyman A, McKeel D, Sumi SM, Crain BJ, Brownlee LM, Vogel FS, Hughes JP, van Belle G, Berg L (1991) The consortium to establish a registry for Alzheimer's disease (CERAD). Part II. Standardization of the neuropathologic assessment of Alzheimer's disease. Neurology 41:479–486

Moore D, Iritani S, Chambers J, Emson P (2000) Immunohistochemical localization of the $P2Y_1$ purinergic receptor in Alzheimer's disease. Neuroreport 11:3799–3803

Nicholas RA, Watt WC, Lazarowski ER, Li Q, Harden K (1996) Uridine nucleotide selectivity of three phospholipase C-activating P2 receptors: identification of a UDP-selective, a UTP-selective, and an ATP- and UTP-specific receptor. Mol Pharmacol 50:224–229

Nitsch RM, Slack BE, Wurtman RJ, Growdon JH (1992) Release of Alzheimer amyloid precursor derivatives stimulated by activation of muscarinic acetylcholine receptors. Science 258:304–307

Nitsch RM, Deng M, Growdon JH, Wurtman RJ (1996) Serotonin 5-HT_{2a} and 5-HT_{2c} receptors stimulate amyloid precursor protein ectodomain secretion. J Biol Chem 271:4188–4194

Pearson RC, Powell TP (1989) The neuroanatomy of Alzheimer's disease. Rev Neurosci 2:101–121

Sadot E, Gurwitz D, Barg J, Behar L, Ginzburg I, Fisher A (1996) Activation of m1 muscarinic acetylcholine receptor regulates tau phosphorylation in transfected PC12 cells. J Neurochem 66:877–880

Selkoe DJ (2001) Alzheimer's disease: genes, proteins, and therapy. Physiol Rev 81:741–766

Tsang SW, Lai MK, Kirvell S, Francis PT, Esiri MM, Hope T, Chen CP, Wong PT (2006) Impaired coupling of muscarinic M(1) receptors to G-proteins in the neocortex is associated with severity of dementia in Alzheimer's disease. Neurobiol Aging 27:1216–1223

Tsang SW, Pomakian J, Marshall GA, Vinters HV, Cummings JL, Chen CP, Wong PT, Lai MK (2007) Disrupted muscarinic M1 receptor signaling correlates with loss of protein kinase C activity and glutamatergic deficit in Alzheimer's disease. Neurobiol Aging 28:1381–1387

Tung EK, Choi RC, Siow NL, Jiang JX, Ling KK, Simon J, Bernard EA, Tsim KW (2004) $P2Y_2$ receptor activation regulates the expression of acetylcholinesterase and acetylcholine receptor genes at vertebrate neuromuscular junctions. Mol Pharmacol 66:794–806

Whitehouse PJ, Price DL, Struble RG, Clark AW, Coyle JT, Delon MR (1982) Alzheimer's disease and senile dementia: loss of neurons in the basal forebrain. Science 215:1237–1239

Wirkner K, Gunther A, Weber M, Guzman SJ, Krause T, Fuchs J, Koles L, Norenberg W, Illes P (2007) Modulation of NMDA receptor current in layer V pyramidal neurons of the rat prefrontal cortex by P2Y receptor activation. Cereb Cortex 17:621–631