The effect of prolyl oligopeptidase inhibition on extracellular acetylcholine and dopamine levels in the rat striatum

THE EFFECT OF PROLYL OLIGOPEPTIDASE INHIBITION ON EXTRACELLULAR ACETYLCHOLINE AND DOPAMINE LEVELS IN THE RAT STRIATUM

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ABSTRACT

Prolyl oligopeptidase (PREP, EC 3.4.21.26) inhibitors have potential as cognition enhancers, but the mechanism of action behind the cognitive effects remains unclear. Since acetylcholine (ACh) and dopamine (DA) are known to be associated with the regulation of cognitive processes, we investigated the effects of two PREP inhibitors on the extracellular levels of ACh and DA in the rat striatum using in vivo microdialysis. KYP-2047 and JTP-4819 were administered either as a single systemic dose (50 μmol/kg ~ 17 mg/kg i.p.) or directly into the striatum by retrodialysis via the microdialysis probe (12.5, 37.5 or 125 μM at 1.5 μl/min for 60 min). PREP inhibitors had no significant effect on striatal DA levels after systemic administration. JTP-4819 significantly decreased ACh levels both after systemic (by ~25%) and intrastriatal (by ~30-50%) administration. KYP-2047 decreased ACh levels only after intrastriatal administration by retrodialysis (by ~40-50%) when higher drug levels were reached, indicating that higher brain drug levels are needed to modulate ACh levels than to inhibit PREP. This result does not support the earlier hypothesis that the positive cognitive effects of PREP inhibitors in rodents would be mediated through the cholinergic system. In vitro specificity studies did not reveal any obvious off-targets that could explain the observed effect of KYP-2047 and JTP-4819 on ACh levels, instead confirming the concept that these compounds have a high selectivity towards PREP.

Keywords: acetylcholine, dopamine, prolyl oligopeptidase, microdialysis, rat

1. INTRODUCTION

Prolyl oligopeptidase (PREP, EC 3.4.21.26, also known as PO, PEP or POP) is a serine protease which preferentially hydrolyses peptides shorter than 30 amino acids at the carboxy terminal of L-proline (Cunningham and O'Connor, 1997). PREP is widely distributed throughout the mammalian body, but the highest activities are generally found in the brain (Agirregoitia et al., 2005, Irazusta et al., 2002, Myöhänen et al., 2007, Venäläinen et al., 2004). Despite intensive research, the main physiological function of PREP has remained unclear. However, recent findings have markedly increased interest in PREP as a drug target; the enzyme has been associated with neuropathological conditions such as accelerated α-synuclein aggregation (Brandt et al., 2008), as well as with several normal neuronal processes e.g. regulation of synaptic plasticity (Di Daniel et al., 2009, Szeltner et al., 2010) and growth-cone development (Di Daniel et al., 2009). These findings, as well as experimental behavioral data from animal studies (for review, see Männistö et al., 2007), point to a role of PREP in neurodegenerative disorders. PREP has also been postulated to modulate the inflammatory response (Gaggar et al., 2008, Tenorio-Laranga et al. 2010) and the generation of the thymosin β4 derived angiogenesis promoting peptide *N*-acetyl-seryl-aspartyl-lysyl-proline (AcSDKP) (Cavasin et al., 2004, Tenorio-Laranga et al., 2009, Myöhänen et al. 2011). Furthermore, novel functions for PREP in intracellular trafficking, sorting and protein secretion (Schulz et al., 2005) as well as in the regulation of the phosphoinositide pathway have been proposed (Schulz et al., 2002, Williams et al., 1999). Interestingly, some of these putative functions seem to be difficult to account for solely by PREP's peptidase activity.

Thus far, the most extensive in vivo evidence has associated PREP with the regulation of cognitive processes, since PREP inhibitors have been shown to improve memory and learning in several different animal models. In rodents, PREP inhibitors can reverse scopolamine induced amnesia in passive avoidance test (Morain et al., 2002,Toide et al., 1995a), in elevated Y-maze (Morain et al.,

2002) and in Morris water maze (Jalkanen et al., 2007). Furthermore, JTP-4819, a potent PREP inhibitor (Toide et al., 1995a), has shown beneficial cognitive effects in passive avoidance test and Morris water maze after the middle cerebral artery occlusion (Shinoda et al., 1996) or the ibotenate lesion of Nucleus basalis magnocellularis (Shinoda et al., 1999). Moreover, JTP-4819 has also alleviated age related memory deficits in rats (Toide et al., 1997).

Despite the fairly extensive evidence pointing to positive behavioral effects of PREP inhibitors on learning and memory, the mechanism mediating the cognitive effects has remained elusive. Many PREP substrates, such as substance P (SP), neurotensin (NT), arginine-vasopressin (AVP), bradykinin, thyrotropin-releasing hormone (TRH) and oxytocin, are involved in learning and memory processes (Cunningham and O'Connor, 1997, Huston and Hasenohrl, 1995, Kovacs and De Wied, 1994). Since PREP is able to cleave these proline-containing peptides in vitro, it has been postulated that PREP inhibitors would reduce the breakdown and increase the brain levels of these memory enhancing peptides in vivo as well (Bellemere et al., 2003, Bellemere et al., 2005, Shinoda et al., 1996, Toide et al., 1995b, Toide et al., 1996). Based on this hypothesis, the cognitive effects of PREP inhibitors have been attributed to direct receptor-mediated actions of the peptides. Alternatively, increased levels of these peptides could stimulate the release of neurotransmitters associated with learning and memory.

Acetylcholine (ACh) is one of the key transmitters in cognitive processes (for recent reviews, see Deiana et al., 2011, Graef et al., 2011, Micheau and Marighetto, 2011), and many putative substrates of PREP are involved in the regulation of cholinergic neurotransmission. For example, increased levels of TRH (Toide et al., 1993) and SP (Perez et al., 2007, Vlasova and Dolgopol'skii, 2000) have been shown to increase ACh efflux in the rat brain. However, little is known about the effects of PREP inhibitors on brain ACh levels. JTP-4819 (1 or 3 mg/kg p.o.) increased extracellular ACh levels significantly but not dose-dependently in the frontal cortex and hippocampus of young rats

(Toide et al., 1995a), but in 24 month old rats, the effect was weaker. The authors hypothesized that JTP-4819 had increased the brain levels of SP, TRH or AVP and in this way increased ACh release. However, this proposed mechanism of action has not been confirmed. Surprisingly, other PREP inhibitors have not been tested for their ability to modulate brain ACh levels.

In addition to ACh, dopamine (DA) is associated with learning and memory (for review, see Myhrer, 2003). Dopaminergic neurotransmission has been related to response selection and habit learning in the rat. As in the case of ACh, PREP inhibitors could theoretically regulate DA release via increased brain levels of PREP substrates. For example, NT exists in close spatial association with DA, and it can induce either an increase or a decrease in DA release depending on the brain area where it is injected (for review, see St-Gelais et al., 2006). The effect of PREP inhibition on brain DA levels has not been evaluated, although DA controls motor functions and behavior. For example, the mesolimbic pathway is involved in the regulation of emotion, addiction and the reward system and the nigrostriatal pathway regulates voluntary movements.

This study was designed to examine the effects of two model PREP inhibitors, KYP-2047 and JTP-4819, on the extracellular ACh and DA levels in the rat striatum. Striatum was selected as the target tissue for the following reasons. The cholinergic system in the striatum is involved in a diverse set of cognitive functions through interactions with other neurotransmitter systems such as the dopaminergic system (for review, see Havekes et al., 2011). In addition, there is emerging evidence from different behavioral tasks suggesting that the striatal cholinergic system undergoes important interactions with the hippocampal cholinergic system e.g. in place and response learning (Chang and Gold, 2003, Pych et al., 2005a, Pych et al., 2005b), and PREP has been associated with these functions. Furthermore, endogenously produced ACh in the striatum is crucial for the induction of corticostriatal long term potentiation (LTP) (Calabresi et al., 1999). ACh and DA are known to regulate each other's release in striatum (Abercrombie and DeBoer, 1997, Brady et al., 2008, Jeon et

al., 2010, Lehmann and Langer, 1983, Millan et al., 2004, Smolders et al., 1997, Threlfell et al., 2010). Finally, PREP protein is widely expressed in the striatum and PREP has been reported to colocalize with striatal ACh (Myöhänen et al. 2008) suggesting an interaction between ACh and PREP.

In the present study, the decrease in ACh levels that did not correlate with the inhibition of PREP activity was observed. In an attempt to elucidate the possible mechanism of action involved in this effect, also the specificities of KYP-2047 and JTP-4819 against several pharmacological targets were studied in vitro.

2. MATERIALS AND METHODS

2.1 Animals

Male Han/Wistar rats, supplied by the Laboratory Animal Centre of the University of Eastern Finland (Kuopio, Finland) were housed in stainless steel cages and kept on a 12-h light/12-h dark cycle at an ambient temperature. The animals were 9 weeks old and weighed approximately 280 g at the beginning of the studies. Animals had free access to pelleted food (Teklad 2016S, Harlan Laboratories Inc, Indianapolis, USA) and tap water. All procedures with the animals were performed according to the appropriate European Community Guidelines and reviewed by the Animal Ethics Committee at the University of Eastern Finland, and approved by the local provincial government (license number ESLH-2008-08303-Ym23). The animal welfare 3R principles (replacement, refinement and reduction) were followed.

2.2 Treatments

Two highly potent PREP inhibitors, KYP-2047 (4-phenylbutanoyl-L-prolyl-2(*S*)-cyanopyrrolidine) and JTP-4819 {(*S*)-2-[[(*S*)-2-(hydroxyacetyl)-1-pyrrolidinyl]carbonyl]-N(phenylmethyl)-1-pyrrolidinecarboxamide}, were used in these studies. Both compounds were synthesized at the University of Eastern Finland as previously described (Jarho et al., 2004, Venäläinen et al., 2002). JTP-4819 was dissolved in saline, and KYP-2047 in saline containing 5% Tween® 80. Controls received 10 ml/kg saline containing 5% Tween® 80. The dose for the systemic administration for both drugs was 50 µmol/kg i.p. (approximately 17 mg/kg). The dose was selected so that a high degree (60-100%) of brain PREP inhibition was achieved with both compounds throughout the experiment (Jalkanen et al., 2011).

ACh levels were also determined after administration of PREP inhibitors directly into the brain by retrodialysis. The perfusate contained 12.5, 37.5 or 125 µM of KYP-2047 or JTP-4819, and it was

introduced via the microdialysis probe for 60 min with a flow rate of 1.5 µl/min. In this method, the dose delivered into the brain is determined by the microdialysis set-up and the relative recovery, which is mainly dependent on the physicochemical properties of the drug (Chaurasia et al., 2007). KYP-2047 has approximately twice as high an in vivo recovery as JTP-4819 in a similar experimental setting (18.4% vs. 9.5%, respectively) (Jalkanen et al., 2011). Therefore, the maximal periprobe extracellular concentrations at the end of the 60 min retrodialysis were approximately 2-20 µM for KYP-2047 (from 12.5 µM × 0.184 to 125 µM × 0.184) and 1.2-12 µM for JTP-4819 (from 12.5 µM × 0.095 to 125 µM × 0.095). Thus, the administration of the lowest concentration by retrodialysis resulted in similar extracellular drug concentrations in the striatum as after the systemic administration of 50 µmol/kg (0.7 µM and 1.3 µM for KYP-2047 and JTP-4819, respectively). The two highest concentrations were chosen in order to assess the dose-dependency of the ACh effect.

In the retrodialysis, KYP-2047 and JTP-4819 were dissolved in saline containing 5% Tween® 80 and diluted to the final concentration with the Ringer's solution (see Microdialysis in conscious rats). After 60 min retrodialysis, the perfusate was changed back to the PREP inhibitor-free perfusate. During the 60 min retrodialysis, controls received perfusate containing the same amount of Tween® 80 in saline as did the PREP inhibitor treated animals (0.00625% v/v Tween® 80 in Ringer's solution for 60 min).

2.3 Microdialysis in conscious rats

Extracellular ACh and DA levels were determined in two separate microdialysis studies with a similar surgical procedure. First, the rats were anesthetized with chloral hydrate (350 mg/kg i.p.; Sigma Chemical Co, St. Louis, MO, USA) and an intracerebral guide cannula (MAB 6.10.IC, AgnTho's AB, Lidingö, Sweden) was implanted stereotaxically into the striatum and fixed to the skull using anchor screws and dental cement. The coordinates measured from bregma (from top of the skull) were AP +0.5 mm; L -3.0 mm; DV -3.8 mm. A single subcutaneous dose of buprenorphine

(0.02 mg/kg; Schering-Plough, Belgium) was given to relieve any postoperative pain, and the animals were allowed to recover from the surgery for 5-6 days in individual cages.

The animals were moved to the microdialysis bowls (CMA 120, CMA Microdialysis, Solna, Sweden), and the microdialysis probe (MAB 9.10.4; 4 mm exposed membrane, 6 kDa cut-off, membrane outer diameter 0.6 mm, AgnTho's AB) was inserted into the brain through the guide cannula. The probe was perfused with Ringer's solution (138 mM NaCl, 1.3 mM $CaCl_2$, 5 mM KCl, 1 mM $MgCl_2 \cdot 6H_2O$, 11 mM $NaHCO_3$, 1 mM $NaH_2PO_4 \cdot H_2O$, and 11 mM *D*-glucose, pH 7.4) (Benveniste and Huttemeier, 1990) at a flow rate of 1.5 µl/min.

In the ACh study, the perfusate was changed into Ringer's solution containing 100 nM of the acetylcholinesterase (AChE) inhibitor, neostigmine after a 12 h wash out period. Before drug administration, 20 min baseline samples were collected for 180 min (ACh) or 120 min (DA) to ensure stabile baselines. After the baseline collection, the appropriate PREP inhibitor or vehicle was administered either intraperitoneally or directly into the brain by retrodialysis, and the dialysate was collected for 5 hours as 20 min fractions. In the DA experiment, 5 µl of antioxidant solution (containing 1mM of oxalic acid, 0.1 M of acetic acid and 3.0 mM of cysteine in water) were added to the tubes to prevent DA oxidation. The dialysates were immediately frozen (-20 °C) and later stored at -80 °C until analyzed within two weeks of collection.

2.4 Analytical

ACh levels in the microdialysates were measured with a highly sensitive liquid chromatography/tandem mass spectrometry (LC/MS/MS) method as described earlier (Keski-Rahkonen et al., 2007). DA levels and the levels of its metabolites homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC) were measured with a high performance liquid

chromatography/electrochemical detection (HPLC/ECD) method as described earlier (Vihavainen et al., 2008).

2.5 Probe verification

After the microdialysis experiments, randomly selected rats were deeply anaesthetized with pentobarbital and then decapitated. The brains were rapidly removed and frozen for 30 seconds in isopentane cooled with dry ice. To verify the location of microdialysis probes, the brains were cut into 30 µm slices using a cryostat (Bright OTF/AS Cryostat, Bright Instrument Co Ltd, Huntingdon, England). The sections were then stained with 0.5% cresyl violet as described by Paxinos and Watson (2007). All examined probes were correctly located in the striatum (data not shown).

2.6 Specificity in vitro

The binding of JTP-4819 and KYP-2047 on a broad variety of pharmacological targets was screened commercially (GenSEPII profile, Caliper Life Sciences, Hanover, USA). The in vitro binding assays of 70 pharmacological targets (Table 1) were carried out in duplicate at a concentration of 10 µM, which is approximately 10-15-times higher than the in vivo brain unbound extracellular C_{max} measured after a single i.p. dose of 50 µmol/kg of JTP-4819 or KYP-2047 (Jalkanen et al., 2011).

In addition, the inhibitory effect of JTP-4819 and KYP-2047 (10 - 100 µM) against several serine proteases and proline-specific proteases were assessed with colorimetric or fluorometric standard methods (Table 2).

2.7 Data and statistical analysis

The mean of last four (ACh) or three (DA and metabolites) baseline samples was calculated for each animal as a baseline value (= 100%) and the alterations of extracellular transmitter concentrations in dialysates after PREP inhibitor treatment were expressed as a percentage of this value. Transmitter

concentrations were not corrected for recovery. A mixed model was used to assess the difference between groups in ACh and DA levels at different time points (time, group and their interaction as fixed effects and rat number as random effect) using SPSS for Windows 14.0.1 software (SPSS Inc., Chicago, USA). The differences with p-values <0.05 were considered statistically significant.

Results are presented as the group means ± S.E.M.

3. RESULTS

3.1 Acetylcholine levels

The mean baseline extracellular ACh level in rat striatum was 12 ± 2 nM. KYP-2047 had no effect on the ACh levels after the systemic administration (Fig. 1). When administered directly into the brain by retrodialysis, the ACh levels decreased by 40-50% compared to control group during the 60 min drug infusion at the two higher concentrations (37.5 and 125 μM) of KYP-2047, but the difference was statistically significant only in the 37.5 μM concentration group ($p<0.05$) (Fig. 2). The ACh levels recovered slowly after the treatment; 240 min after the termination of the drug infusion, the ACh levels were still 30% below the baseline. In addition, the lowest drug concentration (12.5 μM) tended to decrease ACh levels (by ~35%), but more slowly.

JTP-4819 decreased ACh levels maximally by 25% after systemic administration ($p<0.05$ at 300 min, and $p=0.053$ at 280 min) (Fig. 1). In the retrodialysis, all JTP-4819 concentrations decreased ACh levels by 30-50%. Due to the small group sizes, the difference vs. control group was statistically significant only at 20 min (12.5 μM) and 100 min (125 μM) (Fig. 2). The maximum effect (40-50% decrease) was observed 80-100 min after the onset of the retrodialysis. The ACh levels reverted back close to the baseline values within 180 min after the ending of the retrodialysis.

3.2 Dopamine, DOPAC and HVA levels

The mean baseline DA level in rat striatum was 2.7 ± 0.3 nM. There was a trend (ns) towards decreased DA levels in both treatment groups; the maximum effect (~30% decrease) was observed at 140 and 240 min after administration of KYP-2047 and JTP-4819, respectively (Fig. 3). However, DA levels were slightly decreased (by 10-15%) in control animals as well. There were no differences in DOPAC and HVA levels between groups.

3.3 Specificity in vitro

The GenSEPII screening profile did not reveal any effect of KYP-2047 or JTP-4819 on the pharmacological targets that could explain the observed changes in the ACh levels (Table 1). Overall, no significant inhibition of specific radioligand binding was observed by KYP-2047 and JTP-4819, and according to the preset criteria of the GenSEPII profile (the compound should show inhibition of specific radioligand binding by >50%), no off-targets were found.

At 10 µM, KYP-2047 or JTP-4819 did not exert any significant inhibitory effect against several serine proteases and proline-specific proteases (Table 2). About 20 % inhibition of lysosomal prolyl carboxypeptidase activity was seen at 10 µM for each of the PREP inhibitors KYP-2047 and JTP-4819.

4. DISCUSSION

Behavioral studies in rodents indicate that PREP inhibitors have beneficial effects on cognition, but the mechanism remains unclear. Surprisingly, it has not been confirmed whether these effects are associated with ACh and DA, which are known to regulate cognitive processes. Therefore, the effects of two model PREP inhibitors, KYP-2047 and JTP-4819, on the extracellular ACh and DA levels in the rat striatum were assessed in this study.

Both KYP-2047 and JTP-4819 decreased extracellular ACh levels, which does not support the earlier hypothesis suggesting that the positive effects of PREP inhibitors on cognition would be mediated through increased ACh levels (Shinoda et al., 1999, Toide et al., 1995a, Toide et al., 1997). JTP-4819 decreased ACh levels both after systemic and intrastriatal administration by retrodialysis, whereas KYP-2047 decreased the levels only after the intrastriatal treatment. The observed difference between treatments may be attributable to the different pharmacokinetic characteristics of these molecules; JTP-4819 has been shown to yield higher brain extracellular concentrations than KYP-2047 after an equimolar single intraperitoneal dose (Jalkanen et al., 2011), but after intrastriatal administration by retrodialysis KYP-2047 reached higher maximal extracellular concentrations than JTP-4819 (see chapter 2.2 for calculations).

It is difficult to attribute the decrease in ACh levels after KYP-2047 and JTP-4819 treatments to inhibition of PREP peptidase activity. Firstly, recent studies have found no evidence that PREP would regulate the brain levels of peptides (e.g. SP, TRH or AVP) that are associated with the regulation of ACh release (Brandt et al., 2005, Jalkanen et al., 2011, Nolte et al., 2009, Tenorio-Laranga et al., 2009). Furthermore, one would expect an increase in the ACh release if PREP had elevated the levels of these neuropeptides. Secondly, the degree of brain PREP inhibition by KYP-2047 and JTP-4819 and their effect on ACh levels are not parallel; both compounds are known to inhibit brain PREP by 80-100% within 10-30 min after a single dose of 50 µmol/kg i.p. (Jalkanen et

al., 2011). In the present study, however, only JTP-4819 decreased ACh levels at this dose. When the compounds were administered directly into the brain by retrodialysis, both rapidly decreased ACh levels but no clear dose-response was observed. This may be explained by the fact that the maximal extracellular concentrations in the periprobe tissue have been considerably higher after intrastriatal administration than after the systemic administration. Furthermore, the ACh levels recovered close to the baseline within 4 h after the termination of the retrodialysis, although the PREP enzyme around the probe is anticipated to remain fully inhibited for at least 6-8 h after all three retrodialysis concentrations with these long-acting tight binding inhibitors. In summary, the decrease in ACh levels does not correlate with the degree of PREP inhibition, and higher brain drug levels are needed to modulate ACh levels than to inhibit PREP. This may point to an off-target mechanism for KYP-2047 and JTP-4819, or to a mechanism beyond the catalytic activity of PREP. Indeed, it has been suggested that PREP regulates certain intracellular functions via cytosolic protein-protein interactions rather than via its peptidase activity (Di Daniel et al., 2009, Schulz et al., 2005). PREP inhibitors might modulate these functions by causing conformational changes in the tertiary structure of the enzyme and thus perturb the protein-protein interactions (Fuxreiter et al., 2005).

The spatial associations between PREP and ACh do not support a major role for PREP in the regulation of ACh release (Myöhänen et al., 2008, Peltonen et al., 2011). Immunohistochemical studies have shown that there is 26-57% colocalization of PREP protein and the ACh marker choline acetyltransferase (ChAT) in various brain areas. Moreover, ChAT is only partially colocalized with PREP in the cortex, but not in the hippocampus or medial septum. PREP is present in the short cholinergic interneurons in striatum, but not in the long neurons projecting from the septum to the hippocampus, which are known to be important for the ACh-mediated cognitive functions. In striatum, there is approximately 30% colocalization of PREP with ChAT (Myöhänen et al., 2008).

This partial colocalization further supports our findings that the effects of KYP-2047 and JTP-4819 on the striatal ACh levels are not mediated by inhibition of PREP catalytic activity.

The ACh results of this study do not confirm the findings of the only earlier microdialysis study on the effects of PREP inhibition on brain ACh levels (Toide et al., 1995a). In that study, JTP-4819 (1-3 mg/kg = 2.8-8.3 µmol/kg p.o.) significantly but not dose-dependently increased ACh release in the frontal cortex and hippocampus of young and old rats. The difference between the results of that study and our study may be explained by a number of factors. Firstly, different brain areas were investigated. Secondly, we used doses that have been proven to inhibit PREP by 60-80% for 5 h. Most importantly, Toide and co-workers used an extremely high concentration (10 µM) of the AChE inhibitor physostigmine in the perfusate in order to obtain detectable quantities of ACh. Physostigmine is known to elevate brain ACh efflux especially at higher concentrations (Messamore et al., 1993), and this manipulation could well account for the differences between the present report and that of Toide et al (1995a). In recent years, many improvements in the sensitivity of HPLC/MS assays for ACh have been introduced, thus permitting a reduction of the perfusate concentrations of the AChE inhibitors from the micromolar to the present nanomolar range. In the present study, quantifiable ACh levels were reached by the addition of 100 nM of neostigmine, which is the standard protocol nowadays and it is not believed to significantly affect ACh release (Himmelheber et al., 1998, Kehr et al., 2010). It has even been postulated that the addition of low-concentration neostigmine is necessary for assessing the functional changes in the release of ACh (Chang et al., 2006).

As far as we are aware, this is the first report on the effects of PREP inhibitors on brain DA levels. KYP-2047 and JTP-4819 tended to decrease (ns) the extracellular DA levels, but the interindividual variation was high. Moreover, no effect was seen in DOPAC and HVA levels; only the normal fluctuations related to the circadian rhythm was observed (Castaneda et al., 2004), which were

parallel with the minor changes in the DA levels. The lack of effect on DA levels did not encourage us to conduct further studies with the retrodialysis approach.

Although DA and ACh systems exist in close proximity in striatum, the small change seen in dopaminergic transmission can hardly explain the observed changes in the ACh levels. This result is consistent with immunohistochemical studies indicating that in striatum PREP is not present in the dopaminergic fibres (Myöhänen et al., 2008). Furthermore, destruction of dopaminergic neurons in the medial forebrain bundle with 6-hydroxydopamine had no effect on the PREP activity or on its immunoreactivity in striatum, which is the terminal projection area of these dopaminergic neurons (Peltonen et al., 2011). NT, a PREP substrate in vitro, is known to regulate DA release in the brain (St-Gelais et al., 2006), but recent studies have shown that PREP inhibitors have no effect on either the total brain tissue or on the extracellular NT levels (Jalkanen et al., 2007, Jalkanen et al., 2011). In summary, current evidence points to only a minor role for PREP inhibitors in the modulation of the dopaminergic system. Thus, the positive cognitive effects of PREP inhibitors are not likely to be mediated through the dopaminergic system. On the other hand, the lack of dopaminergic effect in the striatum suggests that PREP inhibitors do not have the potential to induce extrapyramidal side effects.

In an attempt to elucidate the possible mechanism of action involved in the decreased ACh levels, an in vitro pharmacological profiling was done for KYP-2047 and JTP-4819. This analysis of 70 pharmacological targets did not reveal any obvious off-targets that could explain the effect on ACh levels. Furthermore, KYP-2047 and JTP-4819 did not exert any inhibitory effect on several serine proteases and proline-specific proteases in vitro at 10 μM concentration. Slight inhibition of lysosomal prolyl carboxypeptidase was observed at 10 μM, which is a 1000-fold higher concentration than needed to fully inhibit PREP in vitro. As far as we are aware, prolyl carboxypeptidase has not been associated with the regulation of ACh levels in the brain. Overall,

these results validate the proposal that KYP-2047 and JTP-4819 have a high selectivity towards PREP.

In humans, plasma AChE activity has been observed to gradually increase after multiple doses of JTP-4819 (Umemura et al., 1997). Theoretically, if this increase holds true in the brain after a single dose as well, it could explain the decrease in the extracellular ACh levels. In the present study, the in vitro screening showed that there was no significant interaction between JTP-4819 or KYP-2047 and AChE (Table 1), i.e. the increase in the AChE activity may be mediated through an indirect mechanism. Further studies are needed to assess the effect of PREP inhibitors on the AChE activity in the rat brain in vivo.

In conclusion, the two model PREP inhibitors, KYP-2047 and JTP-4819, decreased the extracellular ACh levels in rat striatum. This result does not support the earlier hypothesis that the positive cognitive effects of PREP inhibitors would be mediated through increased ACh release. KYP-2047 and JTP-4819 showed no significant effect on brain DA levels, indicating no involvement of dopaminergic mechanisms in PREP inhibitor-induced cognitive effects. In vitro specificity studies did not reveal any obvious off-targets which could explain the effect of KYP-2047 and JTP-4819 on ACh levels.

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Legends for figures

Figure 1: The effect of KYP-2047 or JTP-4819 after systemic administration (50 µmol/kg i.p.) on extracellular ACh levels in rat striatum (n=8-9/treatment) (* $p<0.05$ vs. Control).

Figure 2: The effect of KYP-2047 or JTP-4819 intrastriatal administration by retrodialysis (12.5, 37.5 or 125 µM × 60 min × 1.5 µl/min) on extracellular ACh levels in rat striatum ($ $p<0.05$ 12.5 µM vs. Control; § $p<0.05$ 125 µM vs. Control).

Figure 3: The effect of KYP-2047 or JTP-4819 systemic administration (50 µmol/kg i.p.) on extracellular DA, DOPAC and HVA levels in rat striatum (n=6-7/treatment).

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Fig. 1

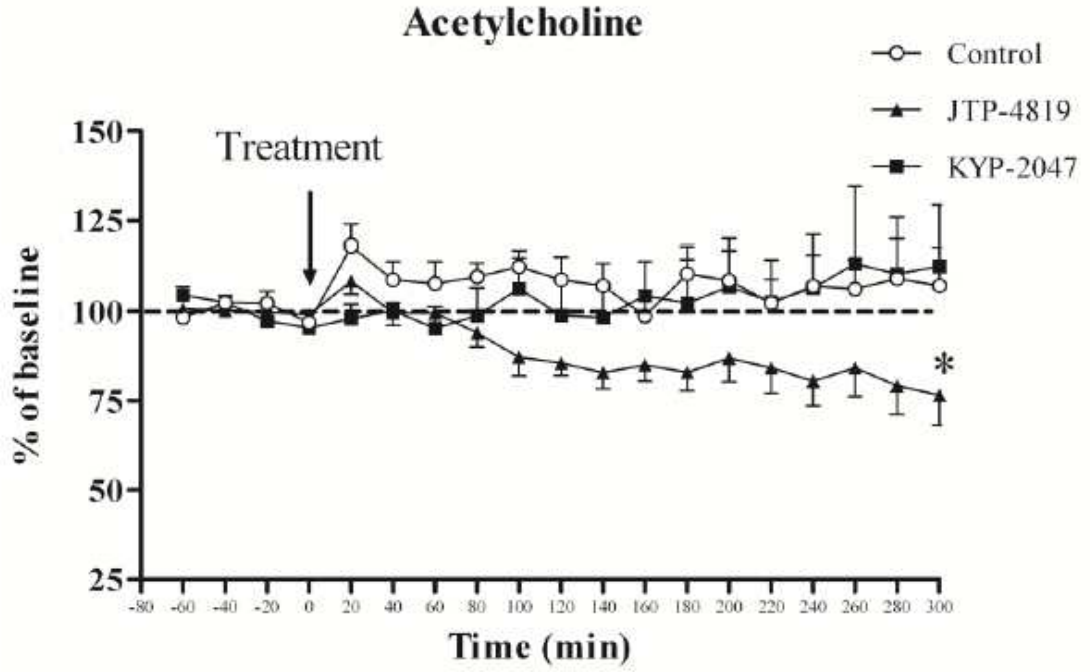
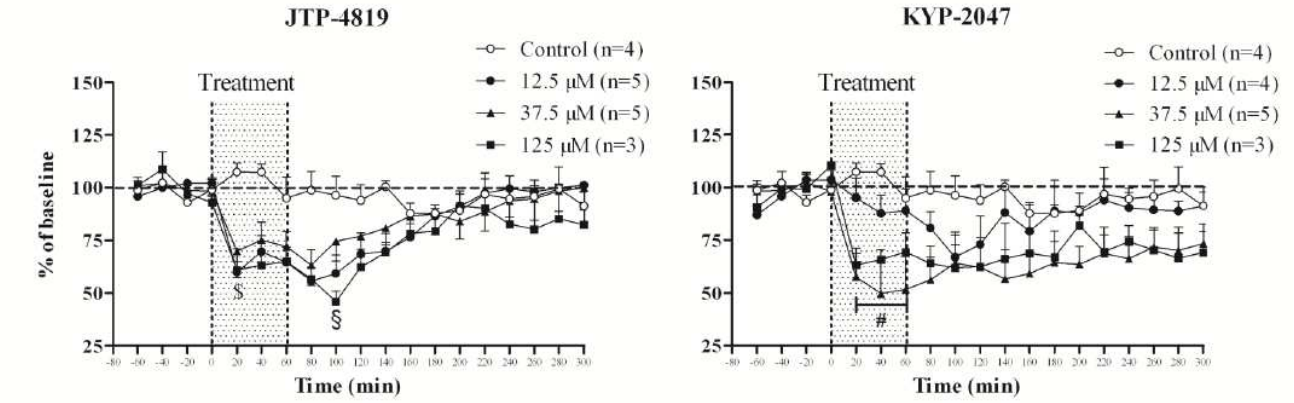

Acetylcholine
Control
JTP-4819
KYP-2047
Treatment
150
125
100
75
50
25
% of baseline
-80 -60 -40 -20 0 20 40 60 80 100 120 140 160 180 200 220 240 260 280 300
Time (min)
*

Fig. 2


JTP-4819
KYP-2047
Control (n=4)
12.5 µM (n=5)
37.5 µM (n=5)
125 µM (n=3)
Treatment
% of baseline
Time (min)
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§
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Fig. 3

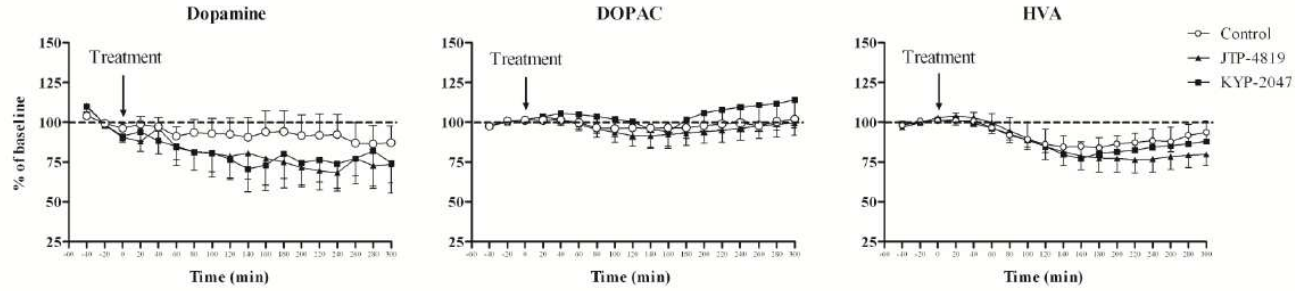
Dopamine
DOPAC
HVA
Treatment
% of baseline
Time (min)
Control
JTP-4819
KYP-2047

Table 1. The binding of KYP-2047 and JTP-4819 on a broad variety of pharmacological targets (GenSEPII profile). Values are expressed as percent inhibition of specific binding and represent the average of two replicate tubes at 10 µM concentration. Inhibition of 50% or greater would be considered significant.

Assay Class	**Assay Name**	**KYP-2047**	**JTP-4819**	**Assay Class**	**Assay Name**	**KYP-2047**	**JTP-4819**
Adenosine	A1	4	11	Leukotriene	LTB4 (BLT)	-7	2
Adenosine	A2A (h)	-6	7	Leukotriene	LTD4	20	19
Adenosine	Transporter(h)	2	3	Muscarinic	M1 (h)	7	-9
Adrenergic	Alpha 1A	20	19	Muscarinic	M2 (h)	-8	9
Adrenergic	Alpha 1B	2	-6	Muscarinic	M3 (h)	8	2
Adrenergic	Alpha 2A (h)	3	3	Muscarinic	M4 (h)	-16	-5
Adrenergic	Alpha 2B	13	-6	Muscarinic	M5 (h)	4	5
Adrenergic	Alpha 2C (h)	0	6	Neurokinin	NK1	16	5
Adrenergic	Beta 1 (h)	-6	-5	Neuropeptide Y	NPY2 (h)	36	11
Adrenergic	Beta 2 (h)	1	7	Nicotinic	a-BnTx Insensitive	-6	1
Angiotensin II	AT1 (h)	-5	-3	Nitric Oxide	NOS	-8	-9
Bradykinin	BK2	-1	5	Norepinephrine	Transporter	6	4
Calcium	Type L	16	-4	Opioid	Delta (h)	17	8
Calcium	Type N	3	-6	Opioid	Mu (h)	8	10
Dopamine	D1 (h)	-4	-5	Phosphodiesterase	PDE4A1A(h)	2	3
Dopamine	D2s (h)	13	25	Phosphodiesterase	PDE5A1 (h)	-1	4
Dopamine	D3	13	20	Potassium	ATP Sensitive	17	10
Dopamine	D4.4 (h)	6	5	Potassium	Ca2+ Activated	9	-2
Dopamine	Transporter	1	-2	Potassium	hERG (I[Kr]) (h)	-11	11
Endothelin	ETA (h)	8	1	Serotonin	5HT1A (h)	25	4
Esterase	Acetylcholine	11	11	Serotonin	5HT1D	5	-5
GABA	Chloride	17	10	Serotonin	5HT2A	-7	-8
GABA	GABA-A Agonist Site	17	-2	Serotonin	5HT2C	-8	-3
GABA	GABA-A BDZ	8	1	Serotonin	5HT3	-8	-9
GABA-B	GABA-B	24	30	Serotonin	5HT4	28	14
Glutamate	AMPA Site	1	33	Serotonin	5HT5A (h)	28	-3
Glutamate	Kainate Site	1	-7	Serotonin	5HT6 (h)	5	0
Glutamate	NMDA Agonist Site	-4	7	Serotonin	5HT7 (h)	10	2
Glutamate	NMDA Glycine Strychnine-insensitive	16	8	Serotonin	Transporter	8	0
Glutamate	NMDA MK801 Site	-11	4	Sigma	S1	9	0
Glutamate	NMDA PCP Site	0	15	Sigma	S2	14	4
Glycine	Glycine Strychnine-sensitive Site	10	-16	Sodium	Site 2	21	-5
Histamine	H1	17	-14	Thromboxane	TXA2 (h)	3	-3
Histamine	H2	20	35				
Histamine	H3	-8	-10				
Kinase, Protein	PKA (h)	13	16				
Kinase, Protein	PKC-alpha (h)	5	7				

(h), human

Table 2. In vitro inhibition of some serine proteases and proline specific proteases by KYP-2047 and JTP-4819.

Target protein (source) EC number	**Substrate (concentration)**	**Method[a]**	**Inhibition (%)[b] KYP-2047[c]**		**Inhibition (%)[b] JTP-4819[c]**	
			10 µM	**100 µM**	**10 µM**	**100 µM**
Serine proteases						
Chymotrypsin (bovine pancreas, Roche) EC 3.4.21.2	Suc-Ala-Ala-Pro-Phe-pNA (250 µM)	20 mM TRIS, 150 mM NaCl, 10 mM $CaCl_2$, pH 8.4	-[d]	<10	-	<10
Subtilisin (B. subtilis Karlsberg, Sigma) EC 3.4.21.62	Suc-Ala-Ala-Pro-Phe-pNA (250 µM)	50 mM TRIS pH 7.6	-	<10	-	<10
Elastase (human leukocytes, Sigma) EC 3.4.21.37	Succ-Ala-Ala-Ala-pNA (250 µM)	0,1 M TRIS pH 8	-	<10	-	<10
Urokinase (uPA) (human, Sigma) EC 3.4.21.73	LpyroGlu-Gly-L-Arg-p-NA.HCl (30 µM)	Joossens et al. 2006	<10	<10	-	-
Tissue plasminogen activator (tPA) (recombinant, Boehringer Ingelheim) EC 3.4.21.6	H-D-Ile-Pro-Arg-pNa.2HCl (1.25 mM)	Joossens et al. 2006	<10	<10	-	-
Thrombin (human plasma, Sigma) EC 3.4.21.5	pyroGlu-Pro-Arg-pNA.HCl (580 µM)	Joossens et al. 2006	<10	<10	-	-
Plasmin (human plasma, Sigma) EC 3.4.21.7	pyroGlu-Pro-Arg-pNA.HCl (580 µM)	Joossens et al. 2006	<10	≈30	-	-
Factor Xa (human plasma, Sigma) EC 3.4.21.6	Boc-D-Arg-Gly-ArgpNA.2HCl (522 µM)	Joossens et al. 2006	<10	<10	-	-
Proline-specific proteases						
Aminopeptidase P (human platelets) EC 3.4.11.9	H-Lys(ABZ)-Pro-Pro-pNA (100 µM)	Stöckel-Maschek et al. 2003	-	<10	-	<10
Dipeptidyl peptidase 2 (human seminal plasma)	Lys-Ala-pNa (1 mM)	Senten et al. 2003	<10	-	<10	-

EC 3.4.14.2						
Dipeptidyl peptidase 4 (human seminal plasma) EC 3.4.14.5	Gly-Pro-pNa (100 µM)	Van Goethem et al. 2011	<10	-	<10	-
Dipeptidyl peptidase 8/9 (bovine testes) EC not yet assigned	Ala-Pro-pNa (300 µM)	Dubois et al. 2010	<10	-	<10	-
Fibroblast activation protein α (human plasma) EC not yet included	Z-Gly-Pro-AMC (250 µM)	0.1 M phosphate buffer pH 7.6	<10	-	<10	-
Carboxypeptidase P (recombinant, Sigma) EC 3.4.17.16	N-Cbz-Glu-Tyr (0.5 mM)	Umetsu et al. 1981	<10	<10	<10	-
Prolylcarboxypeptidase (rat lysosomal) EC 3.4.16.2	N-Cbz-Ala-Pro-Tyr (0.15 mM)	Umetsu et al. 1981	15*	50*	24*	47*
Prolyl imino/aminopeptidase (rat cytosolic) EC 3.4.11.5	Pro-Leu-Gly amide (6 mM)	Turzynski and Mentlein 1990	<10	<10	<10	-
Prolinase (human red blood cells) EC 3.4.13.8/3.4.13.18	Pro-Gly (15 mM)	Uramatsu et al. 2007	<10	<10	<10	-
Prolidase (porcine kidney, Sigma) EC 3.4.13.9	Gly-Pro (25 mM)	Toy et al. 2009	<10	<10	<10	-

[a] In the methods based on Umetsu et al. (1981), Turzynski and Mentlein (1990), Uramatsu et al. (2007) and Toy et al. (2009), the assay was performed in a 48/96 well plate and colorimetric measurement of reaction product was done with Victor^2 or EnVision (PerkinElmer). In all other assays based on chromogenic substrates, colorimetric measurement of reaction product was performed by Spectramax340 (Molecular Devices). Shimadzu RF5000 was used for fluorescence measurements (Aminopeptidase P).
[b] Mean of 2-5 replicates in duplicate
[c] Full inhibiton of PREP (purified enzyme from porcine brain, Venäläinen et al. 2006) by KYP-2047 and JTP-4819 is achieved at 10 nM
[d] (-) Not determined
[*] When N-Cbz-Glu-Tyr (0.15 mM) was used as a substrate, no inhibition was seen by KYP-2047 or JTP-4819 at concentrations up to 100 µM.