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Functional modulation of G-protein coupled receptors during Parkinson disease-like neurodegeneration

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Abstract

G-protein coupled dopamine and metabotropic glutamate receptors (mGlu) can modulate neurotransmission during Parkinson's disease (PD)-like neurodegeneration. PET imaging studies in a unilateral dopamine denervation model (6-OHDA) showed a significant inverse correlation of presynaptic mGlu₄ and postsynaptic mGlu₅ expression in the striatum and rapidly declining mGlu₄ and enhanced mGlu₅ expression in the hippocampus during progressive degeneration over time. Immunohistochemical studies verified the decreased mGlu₄ expression in the hippocampus on the lesion side but did not show difference in mGlu₅ expression between lesion and control side. Pharmacological MRI studies showed enhanced hemodynamic response in several brain areas on the lesion side compared to the control side after challenge with mGlu₄ positive allosteric modulator or mGlu₅ negative allosteric modulator. However, mGlu₄ response was biphasic having short enhancement followed by negative response on both sides of brain. Studies in mGlu₄ expressing cells demonstrated that glutamate induces cooperative increase in binding of mGlu₄ ligands – especially at high glutamate levels consistent with *in vivo* concentration. This suggests that mGlu allosteric modulators as drug candidates will be highly sensitive to changes in glutamate concentration and hence metabolic state. These experiments demonstrate the importance of the longitudinal imaging studies to investigate temporal changes in receptor functions to obtain individual response for experimental drugs.

Graphical abstract

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Keywords

metabotropic glutamate receptor; mGlu₄; mGlu₅; Parkinson's disease; positron emission tomography; pharmacological magnetic resonance imaging

1. Introduction

Recent advances in imaging techniques have enabled investigation of the interactions between superfamily of G protein coupled receptors, including dopamine (DA) and metabotropic glutamate receptors (mGlu), and their associated neurotransmitters; dopamine and glutamate. These receptors, and their modulation in different pathologies including Parkinson's disease (PD), may provide important therapeutic targets.

The primary pathophysiological change that leads to the motor symptoms of PD is a loss of dopaminergic neurons in the substantia nigra pars compacta that modulates the function of the striatum and other basal ganglia structures (Niethammer et al., 2013). The dopamine transporter (DAT) is a membrane-spanning protein, which pumps the neurotransmitter dopamine out of the synapse to cytosol from which it is further transported to vesicles for storage and release. For *in vivo* imaging of DAT function we have previously introduced a series of tropanes (Brownell et al., 1996; Meltzer et al., 1993). Imaging of dopamine function is common in PD and PD models, however far fewer studies have been performed to image changes in mGlu receptors in PD and PD models.

Based on functional mechanisms glutamate receptors are divided into ionotropic and metabotropic glutamate receptors. mGlu function through second messengers and have been target for drug development especially after development of allosteric modulators as drug candidates binding to specific mGlu (Conn et al., 2014). Of the eight mGlu subtypes mGlu₄ and mGlu₅ have received a lot of attention for drug development. mGlu₄ is expressed presynaptically with high density in the striatopallidal synapse (Bogenpohl et al., 2013; Bradley et al., 1999) within the indirect pathway of the basal ganglia. mGlu₄ interacts with the G_{ai/o} subunit of G-protein which couples negatively with adenylate cyclase to inhibit cyclic adenosine monophosphate dependent signal pathways (Ferraguti et al., 1999). mGlu₅ has opposite roles and it is mainly expressed postsynaptically, and coupled with G-protein to stimulate downstream effectors and tyrosine kinases, such as adenylyl cyclase, phospholipase C-β, mitogen-activated protein kinase, and phosphoinositide 3 kinase (Conn and Pin, 1997; Niswender and Conn, 2010; Sheffler et al., 2011). mGlu₅ also has an astrocytic localization (He et al., 2012).

G-protein coupled mGlu modulate intracellular signal transductions and thus regulate the cell physiology (Gubellini et al., 2004; Niswender and Conn, 2010; Pin and Duvoisin, 1995).

Since current PD treatments are based on the replacement of dopamine function and suffer greatly from unwanted side-effects including dyskinesia, the mGlu signaling has become a potential target for drug discovery for treatment of neurological dysfunctions like PD (Johnson et al., 2009). Previous clinical and preclinical studies have shown that dopamine denervation leads to upregulation of the expression of postsynaptic dopamine receptors, which even increases the negative side-effects seen with current dopamine replacement therapy (Aubert et al., 2005; Nguyen et al., 2000; Sanchez-Pernaute et al., 2007). So far relatively little is known about the specific functions, signaling, biodistribution and pharmacology of different mGlu₄ and mGlu₅ subtypes in PD and PD models (Conn and Pin, 1997; Johnson et al., 2009). However, a number of publications support a role for mGlu in development of PD related motor and cognitive dysfunctions (Gasparini et al., 2013; Johnson et al., 2009; Nicoletti et al., 2011).

We explored the functional role of mGlu₄ positive allosteric modulator (PAM) and mGlu₅ negative allosteric modulator (NAM) in the 6-OHDA rat model of PD during fifteen months of progressive degeneration taking advantage of high sensitivity positron emission tomographic imaging (PET) and used our recently developed novel specific radiopharmaceuticals [¹¹C]ML128 (*N*-(4-Chloro-3-[[¹¹C]methoxyphenyl)-2-picolinamide) for mGlu₄ (Kil et al., 2013) and [¹⁸F]FPEB (3-fluoro-5-[(pyridin-3-yl)ethynyl] benzonitrile) for mGlu₅ (Wang et al., 2007).

The 6-OHDA lesion model provides a means to test the effects of mGlu stimulation on the control and on the dopamine denervated side simultaneously. After the last PET imaging studies we investigated the effects of mGlu₄ PAM and mGlu₅ NAM induced regional signal changes recorded as hemodynamic response using the IRON (Increased Relaxivity for Optimized Neuroimaging) technique for measurement of cerebral blood volume (CBV) (Chen et al., 2001; Mandeville, 2012). Regional signal changes were induced by a mGlu₄ ligand, DFMP, (*N*-(4-chloro-3-methoxyphenyl)-2-picolinamide) which is a PAM and a mGlu₅ NAM, MTEP (3-((2-methyl-4-thiazolyl)ethynyl) pyridine). This technique can provide a read-out on receptor functioning, as opposed to receptor numbers as measured by PET (Jenkins, 2012).

2. Materials and Methods

2.1. Animal model

Five male Sprague-Dawley rats (weight between 300 and 450 g) were purchased from Charles River Laboratories (CRL) after lesioning with 6-OHDA and verification with apomorphine. 6-OHDA was delivered unilaterally by stereotactic injection into the substantia nigra on the left side of the rats, leaving the other hemisphere to serve as an internal control.

2.1.1. Animal ethics—All experiments and handlings with the animals were performed in accordance with the National Institutes of Health Guide for the Care and Usage of Laboratory Animals and the usage was approved by the Subcommittee on the Research Animal Care (SRAC) of the Institutional Animal Care and Use Committee (IACUC) for Massachusetts General Hospital (MGH) and Harvard Medical School (HMS).

2.2. In vivo PET imaging studies

2.2.1. Imaging ligands— $[^{11}\text{C}]\text{ML128}$: The radiosyntheses of $[^{11}\text{C}]\text{ML128}$ was performed as previously published (Kil et al., 2013). In brief, $[^{11}\text{C}]\text{CO}_2$ produced by cyclotron was reduced to $[^{11}\text{C}]\text{methane}$ ($[^{11}\text{C}]\text{CH}_4$) which was converted into $[^{11}\text{C}]\text{CH}_3\text{I}$ in gaseous iodination condition. The resultant $[^{11}\text{C}]\text{CH}_3\text{I}$ was transferred in a stream of nitrogen and trapped in a reaction vessel containing the precursor. After completion of the $[^{11}\text{C}]\text{CH}_3\text{I}$ transfer, the reaction vessel was sealed and heated for 5 min. After heating, the reaction mixture was diluted with 1 mL of HPLC eluent, and injected into radio-HPLC using $\text{CH}_3\text{CN}/0.1 \text{ M HCO}_2\text{NH}_4$ solution (45/55) as the mobile phase. The desired product was eluted between 10.4 and 11.0 min. The fraction containing $[^{11}\text{C}]\text{ML128}$ was diluted with 20 mL of water and passed through a C^{18} Sep-Pak Plus cartridge (Waters Corp.) and the cartridge was washed with additional 5 mL of water. The $[^{11}\text{C}]\text{ML128}$ was then eluted from cartridge with 1 mL of ethanol and passed through a sterile filter. The final product was diluted with 9 mL of 0.9% saline to make 10% ethanol solution in saline for animal injection.

$[^{18}\text{F}]\text{FPEB}$: The synthesis of $[^{18}\text{F}]\text{FPEB}$ was conducted through a convenient thermal reaction as a highly specific PET radiotracer for mGlu_5 (Wang et al., 2007). The precursor and standard compounds were prepared by a coupling reaction catalyzed by palladium. Radiosynthesis of $[^{18}\text{F}]\text{FPEB}$ was performed using nitro as a leaving group replaced by $[^{18}\text{F}]\text{fluoride}$ under conventional heating condition.

$[^{11}\text{C}]\text{CFT}$: Radiosynthesis of $[^{11}\text{C}]\text{CFT}$ was performed as previously published (Brownell et al., 1996). ^{11}C -methyl iodide was produced in an automated system following proton bombardment of N_2 containing 0.5% O_2 to yield $^{11}\text{CO}_2$. The $^{11}\text{CO}_2$ was dried under a stream of N_2 gas by heating. The $\text{AlLiO}-^{11}\text{CH}_3$ was treated with hydroiodic acid. The resulting ^{11}C methyl iodide was then transferred as a gas using N_2 as carrier to a reaction vial containing N-demethylated precursor in DMSO and the mixture was heated. At last $[^{11}\text{C}]\text{CFT}$ was purified up to 98%.

2.2.2. Experimental procedures—Experimental imaging studies were initiated one month after the lesioning with 6-OHDA. After a rat was anesthetized by gas (maintained at 1.5-2% isoflurane and oxygen flow of 2 L/min throughout the study), the tail vein was catheterized for administration of radiolabeled ligands. The head was adjusted securely in a stereotaxic head holder equipped with a gas inhalation system. The rat was placed ventrally into the scanner at the center of the imaging field, where the spatial resolution is 1.0 mm for the multimodality scanner, Triumph PET/CT/SPECT, TriFoil, Northridge, CA or 1.8 mm for microPET P4, Concorde Microsystems, Knoxville, TN, USA. The respiration rate, pulse oximetry and heart beat were monitored during the whole imaging time using the physiology system that comes with the scanner. In the P4-scanner physiology was measured via heart rate, pulse oximetry and respiration rate using Vet Ox 4800+ (Loveland, CO) and a transmission imaging was conducted using a rotating cobalt-57 point source before administering radioactivity to create maps for attenuation correction. In the Triumph scanner, an X-ray scanning was done following PET imaging to obtain data for the attenuation correction. PET imaging of dopamine transporter function was done using

[¹¹C]CFT (0.8-1 mCi (29.6-37 MBq) i.v., specific activity 1400 mCi/μmol (51.8 GBq/μmol)) to investigate degree of the lesion. Studies on mGlu₄ and mGlu₅ expression were conducted in the same imaging session. mGlu₄ expression was imaged with [¹¹C]ML128 (0.6-0.7mCi (22.2-25.9 MBq) i.v., specific activity 2720 mCi/μmol (100.64 GBq/μmol)) for 60 min followed 2 hours later imaging of mGlu₅ expression with [¹⁸F]FPEB for 90 min (0.5-0.6mCi (18.5-22.2 MBq)i.v, specific activity 1900 mCi/μmol (70.3 GBq)).

PET imaging studies of dopamine transporter function and expression of mGlu₄ and mGlu₅ were repeated 4 times during the 14-months follow-up period to investigate progressive degeneration induced changes.

2.2.3. PET imaging and data processing—The progressive degeneration induced changes were investigated based on comparison of the left-right side differences of binding potential (BP) of [¹¹C]CFT in the striatum and [¹⁸F]FPEB-BP and [¹¹C]ML128 uptake in the striatum and hippocampus.

In this project, altogether fifty-seven PET imaging studies were conducted in the five 6-OHDA-lesioned rats at time-points 1, 5, 10, and 14 months after the 6-OHDA lesion. At each time-point, a series of PET imaging using [¹¹C]CFT, [¹¹C]ML128 and [¹⁸F]FPEB were performed on each animal. Based on the [¹⁸F]FPEB images of each animal, an image template was built with regions of interest (ROIs) drawn on striatum, hippocampus and cerebellum. Then [¹¹C]CFT images and [¹¹C]ML128 images were registered to the template image. After auto-registration by home-made scripts in MATLAB, all registered images were double-checked to confirm precise registration to the template images. After registration the time activity curves were extracted for each ROI from images of [¹¹C]ML128, [¹⁸F]FPEB and [¹¹C]CFT. The cerebellum was used as a reference region to calculate the BP values for [¹⁸F]FPEB and [¹¹C]CFT with SRTM (Simplified Reference Tissue Model (Lammertsma and Hume, 1996). SRTM produced stable results of BP values without arterial blood sampling and time-consuming metabolite measurements. We found, using four different mGlu₅ radioligands, that there was no apparent specific uptake in the cerebellum (Zhu et al., 2007). Another paper by Rook et al., (Rook et al., 2015) claimed some 20 % displacement of [¹⁸F]FPEB by another, cold mGlu₅ ligand. However the time activity curves in the cerebellum in that paper (Fig. 2) show very little specific uptake in the cerebellum other than non-specific uptake that could have been displaced by the cold ligand. Further, mGlu₅ expression measured in rats using protein immunostaining and western blots showed very little mGlu₅ in cerebellum (Romano et al., 1995). Therefore, we used the cerebellum as a reference region. Because of the expression of mGlu₄ in the cerebellum of rats we could not to use it as a reference tissue to analyze mGlu₄ imaging data. Instead the uptake of [¹¹C]ML128 in left- and right-sided striatum and hippocampus was calculated as a percent of the injected dose per cc (%ID/cc or %ID/g), using right sides' data as control.

After all left- and right-sided BP values of [¹⁸F]FPEB and [¹¹C]CFT and tracer uptakes of [¹¹C]ML128 of each rat were obtained the percent differences were calculated to compare the differences of dopamine transporter, mGlu₅ and mGlu₄ expression. The percentage equation was used to calculate as follows:

$$\frac{\text{Left} - \text{Right}}{\text{Right}} \times 100\%$$

2.3. Pharmacological MRI (phMRI) studies

2.3.1. Preparation of animals for phMRI studies—Rats were anesthetized with 1.2% halothane in O₂. The tail vein was catheterized for both contrast agent and drug administration. Body temperature was regulated using a circulating warm water blanket set to 37°C. Blood pressure was measured by using non-invasive blood pressure monitoring with an inflating tail cuffs (Kent Scientific, Torrington, CT). All procedures were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

2.3.2. Reagents in phMRI—Feraheme (paramagnetic iron oxide nanoparticles) was purchased from Amag Pharmaceuticals (Waltham, MA) and used as a contrast agent (10 mg/kg i.v.) to sensitize the images to cerebral blood volume (CBV). DFMPP, an mGlu₄ PAM was synthesized in our laboratory and used (10 mg/kg i.p.) in phMRI studies. MTEP (3-((2-methyl-4-thiazolyl)ethynyl) pyridine), a negative allosteric modulator of mGlu₅ was purchased from Tocris Bioscience and used in water-solution (10 mg/kg i.v.) as mGlu₅ NAM in phMRI studies

2.3.3. Experimental procedures in phMRI—phMRI images were acquired from a 4.7T Oxford magnet with a Bruker console (Billerica, MA) for the mGlu₄ (DFMPP) and mGlu₅ (MTEP) studies. Rats were maintained at 37°C on 1.2% halothane/O₂/Air mixture (30/70%) the same as for preparation. For these studies we followed the same protocol as published earlier for phMRI studies (Chen et al., 2001; Chen et al., 2011; Mandeville et al., 2014). Briefly, a conventional gradient echo sequence with TR/TE of 350/7.5 ms, flip angle of 40 degrees with one average was used to acquire phMRI data using a home-built single-loop transmit/receive coil of 1.5 cm diameter. Twenty-three contiguous coronal slices were obtained for each time point (45 s/image) with in-plane spatial resolution of 0.2 × 0.2 mm² and a 1 mm slice thickness. We acquired four pre-MION baseline images followed by at least 25 post-MION images for the pre-drug baseline. Then, drugs were injected in the magnet during continuous imaging for another 60 min.

2.3.4. Data Analysis in phMRI

2.3.4.1. Image Co-registration: All images were registered onto the same standard brain template for subsequent averaging across animals. The data were resliced to a thickness of 0.5 mm. Since motion during a functional scan was not a consideration due to anesthesia and the use of a head holder, registration parameters were determined for each functional scan using a single full brain volume that was the average of all time points after MION injection but prior to drug injection. These registration parameters were then applied to all time points in the functional data set. Registration between the functional image set and the standard template was performed by adjusting 12 registration variables in a standard affine transformation as generally applied in human studies. The template images were consistent

with the stereotactic coordinate system atlas from Paxinos (Paxinos and Watson, 1986). Then regions of interest (ROIs) can be drawn for any of the regions defined in the atlas.

2.3.4.2. Functional Analysis: Maps of percent CBV changes were obtained by converting signal intensity changes to $R2^*$ on a pixel by pixel basis (Chen et al., 2001; Mandeville et al., 2004) and assuming a linear relationship between $R2^*$ relaxivity and blood volume. For CBV the transverse relaxation rate ($R2^*$) was measured on a pixel by pixel basis using the standard formulation for gradient echo signal, $R2^*(t) = -\ln(S(t)/S(0))/TE$, where TE is the echo time and $S(0)$ is the average value of the signal before MION. Data acquired after injection of MION were converted to percent change in functional CBV (fCBV (V)) using the change in transverse relaxation rate relative to the pre-contrast baseline: $fCBV = V(t)/V(0) = R(t)/R(0) - 1$. Fits were made to the fCBV data using a GLM model and regressors for the positive and negative components using the jip program (<http://www.nitrc.org/projects/jip>). Then maps were made of p-values for the fits for significant changes of CBV corrected for a false-discovery rate of 5% adjusted for the number of pixels.

2.3.5. Statistics in pHMRI—CBV values from regions of interest on the lesioned and intact hemispheres were obtained by integrating the fCBV values over the full width half maximum of the CBV fit to the positive CBV changes. Then these values were compared (lesion vs. intact) using a paired students t-test. Errors reported are standard deviations.

2.4. Immunohistochemical studies

Rat brains were postfixed in 4% paraformaldehyde and cryoprotected in 30% sucrose. The rat brains were sectioned coronally at 30 μ m using a freezing microtome. Free-floating sections were rinsed with phosphate buffered saline (PBS) with 0.3% TritonX-100 and blocked with 10% normal goat serum in PBS. After blocking brain sections were incubated with mGlu₄ (1:500, ab53088, Abcam) and mGlu₅ (1:1000, ab76316, Abcam) antibody overnight. The tissue was then rinsed in PBS with 0.3% TritonX-100 and incubated for 1 hour in biotinylated secondary antibody (1:200, Vector Labs, Burlingame, CA, USA). After secondary antibody sections were incubated for 1 hour in ABC (Vector Labs) solution at RT. Following incubation, sections were rinsed with PBS and were visualized by incubating in 3,3'-diaminobenzidine (DAB) substrate kit (Vector Labs).

To quantify mGlu₄ sections from the lesion and non-lesion side of the hippocampus were counted using the Optical Fractionator probe in the StereoInvestigator software (MBF Bioscience, Williston, VT) for each group (n=3). The region of interest was delineated at 4 $\times$ magnification. A counting grid of 250 $\times$ 250 μ m was placed over the hippocampus. Using 100 $\times$ 100 μ m counting frame mGlu₄ immunoreactive cells were counted in randomly-placed sampling sites with 40 $\times$ magnification.

2.5. Binding assay

CHO-K1 cells expressing mGlu₄ were used for all binding assay protocols. The binding of [³H]ML128 was measured with increased concentration (0, 10 nM, 100 nM, 1 μ M, 10 μ M and 100 μ M) L-Glutamate (Sigma-Aldrich). For the competitive binding 10 nM of [³H]ML128 was used together with increased concentrations of the DFMPF ranging from

0.01 nM to 10 μ M. 50000 freshly harvested CHO CHO-K1 cells expressing mGlu₄ were transferred to test tubes. The HAMS was used as incubation buffer (10% FBS, 100 units/ml of penicillin/streptomycin and 1mM G418). [³H]ML128 (10 nM) was added to the cell with or without test compound on ice and incubated for 30 min at room temperature. Samples were centrifuged at 1200 rpm for 4 min at +4 °C and washed five times with cold Hams cell culture medium in prior of liquid scintillation counting. Samples were lysed by adding 100 μ l of 0.5 % NaOH and heating at +56°C for 30 min. Samples were cooled with ice path and transferred to VWR® Solvent-Saver™ scintillation vials. To obtain binding parameters the scintillation liquid (Perkin Elmer, Optima Gold) was added (7 ml) in prior to counting in a Packard TriCarb Model (1 min/vial). Nonspecific binding was determined using 10 μ M cold ML128 and specific binding was determined extracting the nonspecific binding from total binding. All measurements were done with three parallels and analyzed with GraphPad Prism software.

3. Results

3.1. Longitudinal PET imaging studies

In the PET studies using [¹¹C]CFT (2 β -carbomethoxy-3 β -(4-fluorophenyl) tropane) we found that a unilateral nigral administration of 6-OHDA induced a decrease of dopamine transporter function in the striatum by $71 \pm 5\%$ ($p < 0.001$) measured one month after the lesioning followed by progressive degeneration of $1.19 \pm 0.07\%$ per month as compared to the control side (Figs. 1A, B; Fig. S.1.). Longitudinal imaging of mGlu₄ and mGlu₅ showed that presynaptically expressed mGlu₄ was enhanced in the striatum on the lesioned side ($12.1 \pm 10.8\%$, $p < 0.08$) as an early response to 6-OHDA administration while mGlu₅ expression at that time was decreased ($-12.3 \pm 4.3\%$, $p < 0.005$). However, during the following progressive degeneration mGlu₄ expression decreased $123 \pm 110\%$ ($p < 0.03$) on the lesion side and mGlu₅ expression recovered $46 \pm 48\%$ (p not significant) resulting an inverse significant ($p < 0.01$) correlation between mGlu₄ and mGlu₅ expression in the striatum on the lesion side (Figs. 1A, C; Figs. S.2., S. 3). In the hippocampus mGlu₄ expression was enhanced $18.5 \pm 11.6\%$ ($p < 0.05$) as an early response to 6-OHDA lesioning but declined $102 \pm 82\%$ ($p < 0.02$) during progressive degeneration. mGlu₅ expression was enhanced by $3.7 \pm 2.0\%$ ($p < 0.02$) and did not change significantly during the whole follow up time of 14 months. There were no observable changes in dopamine transporter function or expression of mGlu₄ and mGlu₅ on the control (right) side during the progressive degeneration.

3.2. pHMRI studies

3.2.1. pHMRI studies with mGlu₄ PAM—pHMRI studies were done using the IRON (Increased Relaxivity for Optimized Neuroimaging) technique to investigate the effects of mGlu₄ stimulation on cerebral blood volume (CBV) using mGlu₄ PAM, DFMPP. The mGlu₄ PAM produced a biphasic pattern of CBV changes with a short increase in CBV followed by a longer decrease in CBV. The pattern of increased and decreased CBV varied somewhat around the brain as shown in the maps (Fig. 2). The increased CBV was highest in the subiculum, thalamus, retrosplenial cortex and infralimbic cortex with the negative CBV being strongest in somatosensory and motor cortices and thalamus. A map of the biphasic

responses segregated into positive and negative CBV changes is shown in Fig. 2. Also shown in Fig. 2 are data from another 6-OHDA lesioned animal (not part of the current cohort) following challenge with 2 mg/kg amphetamine showing a robust response to amphetamine on the intact side, and little response on the lesioned side.

3.2.2. pHMRI studies with mGlu₅ negative allosteric modulator—We also conducted studies with the mGlu₅ NAM MTEP. These data showed a pattern that was superficially similar to the PET studies with large uptake in the posterior ventral hippocampal regions and in the anterior striatal and infralimbic cortex region, although the limited spatial resolution of the PET images precludes a more precise comparison with the pHMRI. There were also differences noted with the mGlu₄ PAM pHMRI data. mGlu₅ NAM induced a positive CBV change in the substantia nigra compared to a negative change with the mGlu₄ PAM. Further, there was much less change in medial prefrontal cortex. There were also significant differences between the lesion and control hemispheres showing a much larger increase on the lesion side in the retrosplenial cortex and a larger increase in somatosensory cortex on the lesion side. There were no other significant differences between the lesioned and control hemispheres with the MTEP. Quantitative values are reported in Fig. 3.

3.3. Binding studies in mGlu₄ expressing CHO cells

The binding density of tritium labeled ML128 was enhanced when the mGlu₄ cells were exposed to higher concentrations of glutamate indicating strong positive co-operation between the allosteric binder and glutamate (Fig. 4A). ML128 is known to have PAM capacity to glutamate-activated mGlu₄. Similar effects are found with the binding of PAMs and the co-operative factor is usually characteristic for each drug candidate (Fig. 4B). In addition, the cell studies showed for the first time, that the binding site of ML128 and its structural analog DFMPP is the same specific site of mGlu₄ (Fig. 4C).

3.4. Ex vivo studies

After *in vivo* imaging studies immunohistochemical studies were conducted of mGlu₄ and mGlu₅ expression. Staining for mGlu₄ expression in cortex and hippocampus showed decreased expression on the lesion side (Fig. 5A) supporting the results obtained by PET studies (Fig. 2C). However, immunostaining of mGlu₅ expression did not show any significant change in cortex and hippocampus between the lesion and control side.

4. Discussion

4.1. PET studies of modulation of mGlu₄ and mGlu₅ expression during neurodegeneration

Our experiments support the result that in PD as well as in experimental PD models, the loss of DA-mediated inhibition in the striatum results in excess excitatory transmission (Johnson et al., 2009). In our studies longitudinal dopaminergic degeneration was investigated with PET imaging studies using [¹¹C]CFT which is a selective and sensitive biomarker for dopamine transporter. These studies showed that unilateral (left side) nigral administration of 6-OHDA induced a decrease in dopamine transporter binding by 71±5% in the lesion side followed by progressive degeneration with a rate of 1.19±0.07% per month (Figs. 1A, B).

However, 6-OHDA administration induced an enhanced mGlu₄ expression as an early response as well as a decrease of postsynaptic mGlu₅ expression in the striatum on the lesion side. PET imaging studies of mGlu₄ expression were done with [¹¹C]ML128 and mGlu₅ expression with [¹⁸F]FPEB. After the immediate effect mGlu₄ expression gradually declined during progressive dopaminergic degeneration while mGlu₅ expression recovered. This is the first study to show inverse modulation of mGlu₄ and mGlu₅ expression in the striatum as a result of the unilateral nigral administration of 6-OHDA. mGlu₄ expression was vulnerable also in the hippocampus. As an early response mGlu₄ expression was enhanced 18.5 ± 11.6% in the lesion side as compared to the intact side. However, during the progressive dopaminergic degeneration mGlu₄ expression declined in the hippocampus almost as much as in the striatum on the lesion side (102 ± 82% vs 123 ± 110%). mGlu₅ expression was enhanced about four percent in the hippocampus on the lesion side and did not change during the 14 months follow-up time. It should be noted that the observed changes were only on the left side when the 6-OHDA was administered into the left nigra. The [¹¹C]CFT, [¹¹C]ML128 and [¹⁸F]FPEB binding did not change on the right intact side in the striatum or hippocampus during the follow-up time. Immunohistochemical studies (Fig. 5) supported PET results of decreased mGlu₄ expression on the left lesion side compared to the right control side in the striatum after 14 months of neurodegeneration period.

Our data showed that glutamate levels had a significant effect on drug binding (Fig. 4). However, the extracellular glutamate concentrations are not essential for baseline PET imaging studies in a rat model of PD by two reasons. Firstly, in the PET imaging studies using [¹¹C]ML128 (EC₅₀ of 110 nM) the drug content was 0.5-0.8 nmol/kg and using [¹⁸F]FPEB (K_d = 0.1-0.15 nM) correspondingly 0.7-0.9 nmol/kg while in pHMRI studies the content of DFMPP (EC₅₀ of 80 nM) was 33.3 μmol/kg and MTEP 42.2 μmol/kg. These studies demonstrate that orthosteric glutamate concentration does not effect on the results obtained by PET studies of expression of mGlu_s. Secondly, the extracellular levels of glutamate have been reported to be about 0.5 – 0.6 M in 6-OHDA lesion rat model. Interestingly, there is no significant difference with extracellular glutamate levels between the lesion and control rats including the striatum area (Robelet et al., 2004). In the PET studies only nanomolar levels of the labeled compounds were used so the results obtained (binding values) are not likely affected by endogenous glutamate (Fig. 4). However, if the allosteric modulators are used with higher doses as drug candidates, it is possible that changes in endogenous glutamate levels could alter drug function. In addition, chronic L-dopa treatment has been shown to increase the extracellular glutamate levels in globulus pallidus synapses. Therefore the increased glutamate levels are more likely be indirectly linked to modification of adaptive properties of glutamate synapses and the glutamate receptors' interactions with other neurotransmitter systems. Additionally, it has been demonstrated that mGlu₄ activation could affect excitatory neurotransmission from the STN to the SNc, suggesting that mGlu₄ activation could reduce glutamate release onto dying dopaminergic neurons. mGlu₄ activation at the striatopallidal area reduces GABA release, which is predicted to normalize basal ganglia output. This leads to the hypothesis that mGlu₄ activation may mimic the actions of dopamine in the indirect pathway by inhibiting GABA release at the striatum–globulus pallidus synapse. In addition, the mGlu₅ antagonists or NAMs appear particularly promising for the treatment of L-DOPA-induced dyskinesia, a

frequent invalidating complication of DA replacement therapy that represents an abnormal form of synaptic plasticity. Finally, pharmacological manipulation of mGlu₅ could open up new modalities of neuroprotection for PD and other degenerative diseases of the nervous system, by decreasing excitotoxicity, modulating signaling pathways or through local translation of trophic factors.

4.2. Allosteric modulators as drug candidates

During the last decade there has been an increasing interest of finding new molecules to target mGlu₅ (Nicoletti et al., 2010; Zhang and Brownell, 2012). Competitive ligands bind to the same orthosteric binding site with Glu in the extracellular Venus flytrap domain in a competitive manner, while other ligands bind to the target protein with non-competitive binding mode (Niswender et al., 2010). All of these compounds may modulate the receptor activity as agonists, antagonists, or positive and negative modulators (Layton, 2005; Niswender et al., 2010). The binding affinities correlate with the agent's ability to stabilize the inactive or active receptor conformation (Kew, 2004). The first functional mGlu₅ compounds were analogs of Glu or glycine derivatives, which were targeting the well characterized orthosteric binding site (Gasparini and Spooren, 2007). However, these ligands suffer from poor receptor selectivity as well as from poor ability to cross the blood brain barrier and leave relatively small amount of possibilities for drug optimization (Carroll, 2008).

During the last two decades there has been a great interest to find non-competitive allosteric modulators to overcome the negative issues with competitive agents (Annoura et al., 1996; Conn et al., 2014; Ritzen et al., 2005). Allosteric binding sites are usually located in seven transmembrane domains, which are distinct from the Glu binding site (Williams and Lindsley, 2005). The structurally more diverse transmembrane domain enabled better receptor sub-type selectivity compared to more homogenous Glu binding site (Kew, 2004). In addition, the allosteric binding sites expanded the variety of molecular scaffolds in drug design, which led to advanced drug and imaging agents with better blood brain barrier penetration, better sensitivity, higher affinity and overall advanced drug properties and therapeutic effects compared to the classical orthosteric ligands (Conn et al., 2009; Wang et al., 2007; Hamill et al., 2005; Patel et al., 2007).

In this project we used for PET imaging of mGlu₄ [¹¹C]ML128 with EC₅₀ of 110 nM and [¹⁸F]FPEB to investigate mGlu₅ (K_d = 0.1-0.15 nM). In pHMRI studies we used DFMP with EC₅₀ of 80 nM.

4.3. pHMRI studies of hemodynamic response in 6-OHDA rat model of PD

As seen in Fig. 2, after challenging with positive allosteric modulator of mGlu₄ we used a general linear model fitting to map the regionally increased (Fig. 2A) and decreased CBV (Fig. 2C) areas in the 6-OHDA lesioned animals. There were a number of brain regions that showed similar CBV increases on both the control and lesion sides such as the infralimbic and medial prefrontal cortices, hippocampus and retrosplenial cortex. On the other hand there were regions that showed a much larger response on the lesion side including sensorimotor cortex and temporal cortex. The negative CBV changes are mostly confined to

cortical and thalamic regions and there were no statistically significant differences between the control and lesion side. A similar map is shown for the negative allosteric modulator of mGlu₅, MTEP, in Fig. 3. For comparative purposes, a map of a PET imaging (Fig. 3A) of expression of mGlu₅ using a negative allosteric modulator, [¹⁸F]FPEB is shown overlaid on the MR image verifying enhanced expression in the prefrontal cortex and hippocampus. Although the pHMRI map (Fig. 3B) of MTEP is superficially similar to that of the mGlu₄ PAM there are some major differences. First, there is less activation in the medial prefrontal cortex and thalamus compared to mGlu₄ agonism consistent with prior studies of the distributions of these receptors (Phillips et al., 1997; Romano et al., 1995). Second, there is a much larger increase in the retrosplenial cortex on the lesion side compared to the intact side unlike for mGlu₄ agonism where there is a similar response on both sides. Further, for mGlu₄ PAM there is a negative CBV response in the substantia nigra compared to a positive response for mGlu₅ NAM. There are similar increases on the lesion compared to the intact side in the cortex (SMCx and temporal) as were noted with the mGlu₄ PAM. Unlike for the mGlu₄ PAM, there were no regions that showed decreased CBV (Figs. 3B, C). Plots of the CBV changes in the intact and lesion sides are shown in Fig. 3D.

The response of an indirect metabolic marker like CBV to mGlu_{4/5} agonism or antagonism is difficult to predict. mGlu₄ agonism can lead to inhibition of adenylate cyclase (Pin and Duvoisin, 1995) but not in all brain regions, as well as decreased glutamate release, however in concert with GABA(A) receptor activation there can be an increase in glutamate release (Antflick and Hampson, 2012). Thus, there is not a simple prediction one can make about the effects of such agonism upon hemodynamic markers. Further, most mGlu₄ compounds also show high affinity for mGlu₈ (Nicoletti et al., 2011). The inhibition of adenylate cyclase often (but not exclusively) noted for mGlu₄ agonism would be predicted to lead to decreased CBV as is seen for dopamine D₂/D₃ agonists while the increased adenylate cyclase activity caused by D₁/D₅ agonists leads to increased CBV (Choi et al., 2006; Jenkins, 2012). Therefore, the negative component of the CBV changes noted with the mGlu₄ compound may reflect this aspect of the drug. This does not provide an explanation of the increased CBV component noted with mGlu₄ agonism, but this may be operating through the inhibition of GABAergic output to the pallidum as noted in both monkeys and rats (Bogenpohl et al., 2013; Valenti et al., 2003). Both the PET and immunohistochemistry show decreased mGlu₄ on the lesioned side. The fact that there is an increase in pHMRI signal on the lesioned side means that the pHMRI is measuring functional output from mGlu₄ signaling rather than merely receptor numbers. The fact that this signal increase on the lesioned side is occurring only in sensory and motor cortex is not incongruent with the motor behavioral amelioration produced by mGlu₄ PAMs in prior studies of 6-OHDA lesioned rats (Valenti et al., 2003). As we showed in prior studies, there is decreased signal seen with DA signaling in the sensorimotor cortex in the 6-OHDA model (Fig. 2 and (Nguyen et al., 2000)) thus the decreased inhibition of the pallidum may lead to increased cortical signal with mGlu₄ stimulation. Thus this remains a topic for future study.

There is good evidence to suggest that MTEP can act as a positive allosteric modulator at mGlu₄ in the micromolar range (Mathiesen et al., 2003). mGlu₅ does not couple through adenylate cyclase like mGlu₄, but rather through phospholipase leading to a cascade of signaling (Pin and Duvoisin, 1995). The mGlu₅ agonist, 1S,3R- ACPD, led to constriction of

mouse arterioles in cortical slices (He et al., 2012) suggesting that mGlu₅ antagonists might increase vasodilatation as noted in the CBV data shown in Fig. 3. We note that this effect may be mediated through astrocytic mGlu₅ receptors rather than neuronal mGlu₅ receptors (see Fig. 6; and (He et al., 2012)). mGlu₅ is upregulated on the cortex on the lesion side in 6-OHDA lesioned animals (Nandhu et al., 2011) even though it appears to be down-regulated on the lesion side on the basal ganglia. Thus, this may explain the increased CBV on the lesion side in the cortex with the MTEP shown in Fig. 3.

The results we obtained provide compelling evidence of mGlu ligand induced blood volume changes in different brain areas in normal and lesion side. Unfortunately, there are no other published studies in PD models to compare our results. Two prior papers used mGlu ligands with ph/fMRI. Sloan et al. showed that the MPEP did not block either BOLD or CBV changes in motor cortex caused by hind-paw stimulation (Sloan et al., 2010) but they did not present results for MPEP alone so it is hard to compare to our data. Simon et al. (Simon et al., 2011) examined mGlu₁ and mGlu₅ drugs, but they used a fast-spin echo sequence that would be expected to have about 24 times lower sensitivity than the IRON technique that we used, so again it is hard to compare results. Interestingly, our results showed that there was little evidence of a large response in the basal ganglia except in the anterior dorso-medial caudate-putamen a region associated with innervation of motor cortex. Thus, this demonstrates again that we are most likely seeing “signaling” rather than a read-out of receptor numbers using the phMRI. This makes it a complementary tool to the PET studies.

We have previously published on both decreased pre-synaptic dopamine transporters as well as upregulation of dopamine receptors using combined PET and phMRI studies in both non-human primate and rodent models of PD (Brownell et al., 1998a; Brownell et al., 1998b; Chen et al., 1997; Jenkins et al., 2004; Nguyen et al., 2000). Similar to what was observed here, with the non-selective DA agonist apomorphine, we saw an increase in signal on the lesioned side.

A model for interpretation of the hemodynamic changes is outlined below.

5. Conclusions

These are the first studies to demonstrate an inverse correlation of mGlu₄ and mGlu₅ expression in the striatum during progressive degeneration in the lesion side induced by the unilateral administration of 6-OHDA into the left nigra. As well these are the first studies to demonstrate mGlu₄ PAM and mGlu₅ NAM induced hemodynamic changes in several brain areas in normal and lesion side also demonstrating higher hemodynamic response on the lesion side. These studies showed that, if the allosteric modulators are used with pharmacological doses as drug candidates, endogenous glutamate level has significant effect on drug response. These experiments also demonstrate the importance of the longitudinal in vivo imaging studies to investigate temporal changes in receptor functions to obtain individual response for experimental drugs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Modulation of mGlu₄ and mGlu₅ was found on the lesion side of 6-OHDA rat
- mGlu₄ and mGlu₅ have inverse correlation during progressive striatal degeneration
- mGlu₄ PAM and mGlu₅ NAM induced hemodynamic changes enabling phMR imaging
- PAM and NAM induced signal changes in phMRI are complementary to PET imaging of mGlu₅
- Endogenous glutamate modulates dose response and binding of allosteric modulators

Figure 1. PET imaging studies of modulation of dopamine transporter function and expression of mGlu₄ and mGlu₅ in the brain of 6-OHDA lesioned rat during progressive degeneration
 (A) Distribution of [¹¹C]CFT ([¹¹C]2β-carbomethoxy-3β-(4-fluorophenyl)) on coronal (striatal and hippocampal level) and midstriatal axial slices shows abolished accumulation on the left (lesion) side. Distribution of [¹¹C]ML128 (*N*-(4-chloro-3-[¹¹C]methoxyphenyl)-2-picolinamide) on the same level as above shows enhanced accumulation on the left (lesion) side. Distribution of [¹⁸F]FPEB (3-fluoro-5-[(pyridin-3-yl)ethynyl] benzonitrile) on the same level as above shows decreased accumulation in the striatum and enhanced accumulation in the hippocampus on the left (lesion) side. (B) Exponential decline of dopamine transporter (DAT) binding of [¹¹C]CFT in the striatum is an indication of progressive degeneration with the rate of 1.19 ± 0.07 % per month. (C) Significant inverse correlation of mGlu₄ and mGlu₅ expression was observed in the lesioned striatum during progressive degeneration ($\text{Value}_{\text{mGlu}_5} = (-0.478 \pm 0.477) \times \text{Value}_{\text{mGlu}_4} - 7.34$, $r=0.647$, $p<0.01$). mGlu₄ expression declined in the hippocampus 102 ± 82 % while mGlu₅ expression did not change during progressive degeneration.

Figure 2. phMRI studies in 6-OHDA rat model of PD after challenge of mGlu₄ PAM or amphetamine

There were positive and negative CBV responses to the injection of the mGlu₄ PAM (DFMPP). The initial response was positive as shown in the map (A) which shows a statistical map of the positive CBV response. Note the increased CBV on the lesion side (left) showing cortical upregulation of mGlu₄ signaling. In the thalamus, hippocampus infralimbic, medial pre-frontal and retrosplenial cortices there were no differences between the lesion and non-lesion sides (B). For the positive component of the CBV there were significant (paired T-test) differences between the somatosensory and temporal cortices (*, $p < 0.05$). A map of the negative component of the CBV induced by the mGlu₄ PAM is shown in (C). The negative component of the CBV was more uniform on the lesion and intact sides and the differences were not significantly different. The decomposition of the CBV time course in the thalamus into the negative (blue) and positive components (red) used to make the maps in (A and C) is shown in (D). (E) shows an opposite CBV response after amphetamine induced dopamine release confirming that the dopamine release is abolished from the left lesion side while the intact right side has normal function. Comparing the images (A) and (E) provides a striking evidence of functional difference between glutamatergic and dopaminergic neurosystems.

Figure 3. Maps of mGlu₅ in 6-OHDA lesioned rats

(A) PET image showing the uptake of $[^{18}\text{F}]$ FPEB normalized to the percent of the highest uptake in an animal 14 months after lesioning. There is extensive uptake in the medial prefrontal and infralimbic cortex, striatum as well as in the hippocampus. (B) An averaged map of T-values for significant changes due to stimulation with the mGlu₅ negative allosteric modulator (MTEP). There are significant upregulations of the response on the lesion side in the SMCx and retrosplenial cortex (shown with the red arrows) compared to the intact side. The infralimbic cortex and hippocampus were relatively symmetric. (C) Comparison of fCBV changes induced by the mGlu₅ and mGlu₄ ligands showing only positive fCBV changes with the mGlu₅ compound. (D) Plot of integrated changes in fCBV on the lesion versus the contralateral side (*, $p < 0.05$; paired T-test).

Figure 4. Evidence of cooperative nature of mGlu₄ PAM binding site

(A) Specific binding of 10nM [³H]ML128 to CHO expressing mGlu₄ receptor was determined in the presence of increasing concentration of the natural agonist. Binding of [³H]ML128 without addition of glutamate is set as 1.0 and presented together with SD. (B) The ability of 10 nM cold ML128 or DFMPD to displace the [³H]ML128 (10nM) from its binding site was measured without or increased concentration of natural agonist. The binding of 10 nM [³H]ML128 was set as 1.0 and presented together with SD. (C) Competitive binding assay was done with single dose (10 nM) of hot [³H]ML128 and increased concentration of cold DFMPD. The specific radioactivity of 10 nM [³H]ML128 was set as 100 and presented together with SD. All measurements were performed in three parallels and analyzed with GraphPad Prism.

Figure 5. Representative pictures showing mGlu₄ and mGlu₅ immunostained rat brain Sections from the lesion and control side of the cortex and hippocampus fourteen months after the unilateral administration of 6-OHDA into the left nigra (magnifications $\times 4$, $\times 20$). (a) Arrows indicate mGlu₄ expression area. At that time point mGlu₄ expression had slightly decreased in the lesion side in the cortex and hippocampus of rat brain compared to non-lesion side. (B) Quantitative analysis using a paired t-test showed significant ($p<0.002$) decrease of the mGlu₄ expressing cells on the lesion side of the hippocampus. (C) Immunostaining of mGlu₅ expression did not show a significant difference between lesion and control side.

Figure 6. The synaptic interplay of mGlu₄, mGlu₅ and calcium dependent vasoactive signaling
 Schematic illustration of the major DA and Glu synaptic effects and how these may influence cerebral blood flow. The primary pathophysiological change that leads to the motor symptoms of PD is the loss of dopaminergic neurons in the substantia nigra pars compacta that modulates the function of the striatum and other basal ganglia structures. The L-DOPA is considered the standard of care for providing symptomatic relief for PD patients. However, long-term L-DOPA treatment leads to the appearance of motor complications (Ahlskog and Muentner, 2001). On the other hand, the excessive Glu signaling in the basal ganglia is one of the hallmarks of the progression of PD. The mGlu (especially mGlu₄ and mGlu₅) modulators were considered to be one of the best potential drug candidates since the striatum also sends direct projections to the substantia nigra pars compacta, which leads to a parallel increase in Glu release, GABA signaling and contributes to the progressive neuronal degeneration also via an excitotoxic mechanism (Gasparini et al., 2013). The hyperactive

Glu stimulus demands energy and couples the hyperactivation tightly with blood flow (Lauritzen et al., 2012). It has been suggested that the astrocytes are able to detect the Glu driven synaptic activity and thus modulate the local blood flow (Attwell et al., 2010). In addition, ATP and its derivative adenosine modulates the blood flow (Latini and Pedata, 2001) as does DA acting on both astrocytes and blood vessels (Choi et al., 2006). Together with dopamine transporter, the synaptic contacts possess a variety of neurotransmitter receptors, including both ionotropic and metabotropic glutamate receptors (Beaulieu and Gainetdinov, 2011). Activation of Glu receptors by synaptically released Glu is known to lead to transient and graded increases in intracellular Ca^{2+} concentration (Marambaud et al., 2009). Since the Glu signaling is hyperactive in PD, it is assumed that the inhibition of Glu signaling should decrease the PD symptoms, the synaptic activity and eventually lead to decrease of the local blood flow due to decreased energy demand and Ca^{2+} signaling in pathological site (Attwell et al., 2010; Lauritzen et al., 2012).