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Radiosynthesis of high affinity fluorine-18 labeled GnRH peptide analogues: *in vitro* studies and *in vivo* assessment of brain uptake in rats

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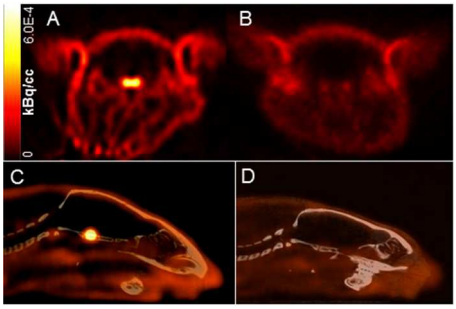

Novel ^{18}F-Gonadotropin releasing hormone peptides have been radiosynthesised with very high affinity for the GnRH receptor and good *in vivo* stability but do not enter the brain as suggested by others.

(Electronic supplementary information (ESI) available)

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Abstract

Gonadotropin releasing hormone (GnRH) is recognized as an important neuromodulator affecting behavior and has been associated with the progression of Alzheimer's disease. The peptide has been shown to have a bidirectional transport through the blood-brain-barrier (BBB), which may account for the cognitive effects of systemically administered GnRH. In this study, four novel ^{18}F-GnRH peptide analogues were synthesized and their *in vitro* and *in vivo* characteristics studied in male rats.

GnRH peptides were assembled by solid-phase peptide synthesis, either as the full length D-Lys^6-GnRH (pyroGlu^1-His^2-Trp^3-Ser^4-Tyr^5-D-Lys^6-Leu^7-Arg^8-Pro^9-Gly^{10}-NH_2) or as D-Lys^6-desGly^{10}-GnRH-NHEt. In all, four GnRH peptide analogues were synthesized and reacted with *N*-succinimidyl-4-fluorobenzoate (SFB) to yield the fluorinated versions. Binding affinities of the analogues were determined in a competitive binding assay for both human and rat GnRH receptors. K_i-values for the GnRH peptides were found to be subnanomolar, with D-Lys^6(FBA)-desGly^{10}-GnRH-NHEt **(7)** being most potent with a K_i-value of around 50 pM for GnRH receptor species.

Radiolabeling was performed using *N*-succinimidyl-4-[^{18}F]fluorobenzoate ([^{18}F]SFB) in 33.3±12.8% isolated decay corrected (d.c.) yield within 1.5 - 2 h. Rat serum stability over 2 h revealed minor degradation (≤ 5%). For *in vivo* studies, ^{18}F-peptides (4-30 MBq)

were injected intravenously via the tail vein into rats and brain uptake was evaluated by means of dynamic PET (2 h) followed by biodistribution studies. PET showed limited or no uptake in brain for the ^{18}F-peptides which predominantly cleared rapidly by renal excretion. Specific binding in the pituitary gland was confirmed for the ^{18}F-peptide, **7**, by blocking with the GnRH agonist buserelin.

Introduction

Gonadotropin-releasing hormone (GnRH) is a neuroendocrine decapeptide (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) synthesized in the neurovascular terminals of the hypothalamus and secreted from the median eminence in a pulsatile manner into the hypophyseal portal blood supply.[1] At the anterior pituitary, GnRH selectively binds to the GnRH receptor (GnRHR) on the membranes of the gonadotroph cells, and, through its binding, stimulates synthesis and release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH).[2]

Traditionally, GnRH has been considered predominantly a hormone central to the control of reproductive physiology. However, extra-hypothalamic GnRH and GnRHR are found in other tissues, including the heart and brain, where their function remains poorly understood.[1,3,4] Overexpression of GnRHR is also found in a number of human cancer cells, both from reproductive and non-reproductive organs. Examples include ovary, endometrium, prostate and breast. In tumors of non-reproductive organs and tissues, some examples are the liver, larynx, pancreas, colon, lymphoma, kidney, skin, blood and brain.[5]

In the mammalian brain, the GnRHR is particularly highly expressed in the hippocampus (3.3 fmol/mg protein), a region related to learning and cognition.[6,7] Interestingly, recent evidence points towards a role of GnRH in modulating cognitive functions in the hippocampus which further might have implications for the progress of dementia.[8-10] To this end, the growing body of evidence supporting this assertion includes the increased prevalence of Alzheimer disease (AD) in women, the correlation of serum gonadotropins with the disease, as well as the decreased incidence and delay of onset in AD patients following GnRH blockage therapy.[4,8,10] Furthermore, preclinical studies conducted in mice where GnRH treatment reduced total brain Aβ1-42 and Aβ1-40, and thus add support to the GnRH-AD connection hypothesis.[11,12] Considering the aforementioned findings, GnRHR located in central nervous system (CNS) could potentially be a target for use in positron emission tomography (PET) for assessment of GnRHR expression and shed light on the GnRH connection in diseases associated with cognitive decline such as AD.

Peptides, with few exceptions, have traditionally not been considered a class of molecules that readily pass the blood-brain-barrier (BBB). This is related to their relative large molecular weight (600-2000 Da) with the abundant amide bonds and ionizable functional groups. Indeed, it is often assumed that peptides and proteins cannot cross the BBB, despite evidence of the contrary.[13] Different classes of peptides have been shown to mediate effects in the CNS and crossing the BBB through active transport; examples include enkephalin, leptin, insulin and tumor necrosis factor-α.[14-16] Encouragingly for this study, an active bidirectional uptake of GnRH into the brain has been suggested by Barrera *et al.* and is further supported by recent literature reports detecting brain uptake

following i.v. administration of I^{125}-labeled GnRH.[17, 18] It should be noted, however, that based on experiments similar to those conducted by Barrera *et al.*, Verheugen *et al.* concluded that the BBB is impermeable to GnRH.[19]

To date, no reports have been published describing fluorine-18 (^{18}F) labeled-peptides that target receptors in the CNS. In light of this, and given the potential for brain uptake of GnRH described in the literature, it is of considerable interest to study ^{18}F-labeled-GnRH peptides in order to investigate if uptake in the brain and binding to hippocampal GnRHR can be imaged. Given its very high sensitivity and the ability to non-invasively obtain tomographic images without depth-limitation, PET imaging is uniquely suited to investigate non-invasively the potential for brain uptake *in vivo* or GnRHR expression in general. We present herein the synthesis of four novel GnRH-peptide analogues, radiolabeling with the PET radionuclide fluorine-18, *in vitro* characteristics (log P and serum stability), competitive binding studies and small animal imaging in healthy rats with emphasis on brain uptake, as well as follow-up biodistribution. We further demonstrate that the radiolabeled peptides are specific towards the GnRHR *in vivo* by co-administration of buserelin, a clinically relevant GnRH agonist and that the investigated fluorine-18 labeled peptides are putatively promising candidates for imaging of GnRHR expression *in vivo*.

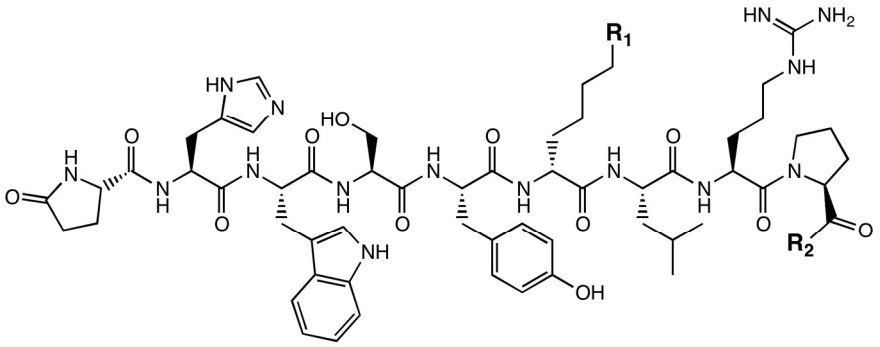
Results and discussion

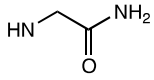
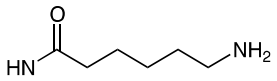
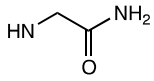
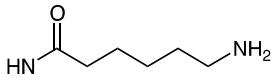
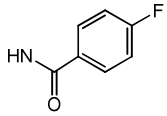
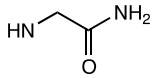
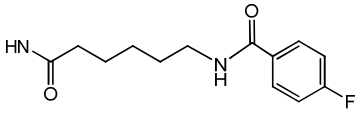
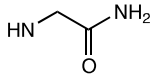
Chemistry. Non-radiolabeled peptides **1**-**4** (Table 1) were synthesized using established Fmoc-protocols. Initially, attempts to synthesize the target peptides using unprotected pyroglutamic acid resulted in a complicated reaction mixture with only trace amounts of

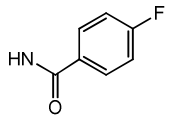
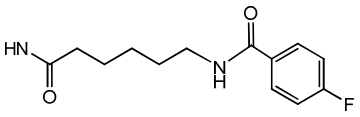
product. Unintentional acylation of the pyroglutamic acid lactam nitrogen by excess HATU-activated amino acid during coupling was found to be the cause (data not show). Therefore, Boc-pyroglutamic acid was chosen for all further preparations. For introduction of [^{18}F]FBA into peptides *via* its activated ester ([^{18}F]SFB), the glycine^{6} in native GnRH was replaced with D-Lys for all analogues. The D-lysine serves as an attachment point for the [^{18}F]FBA and simultaneously increases binding affinity and metabolic stability by stabilizing the folded conformation of the peptides and incorporating an unnatural D-amino acid.[20,21] Peptides **3** and **4** are similar in structure to the potent commercially available GnRH superagonists leuprolide ([D-Leu^{6}, Pro^{9}-NEt]GnRH) and deslorelin ([D-Trp^{6}, Pro^{9}-NEt]GnRH), where the terminal Gly^{10}-amide in native GnRH is replaced by an ethylamide residue (Fujino modification).[21] The addition of Pro^{9}-ethylamide is well documented, and has been shown to give a fourfold increase in affinity compared to the GnRH analogues possessing the single D-Trp^{6} modification.[22] Peptide analogues **2** and **4** were generated by coupling 6-aminohexanoic acid to the ε-nitrogen of D-Lys. The 6-aminohexanoyl amide spacer has been shown to increase stability (*in vitro*) and also gives the peptides a more hydrophobic character as compared to their unmodified counterparts **1** and **3**.[20] The prosthetic group [^{18}F]fluorobenzoic acid ([^{18}F]FBA) was selected for radiolabeling based on its well-documented *in vivo* stability and hydrophobic nature;[23] D-lysine^{6} modified analogues of GnRH can tolerate bulky hydrophobic groups with little or no loss of binding affinity, and a general increase in lipophilicity of the peptides was perceived to be beneficial for brain uptake compared to a more hydrophilic prosthetic group (such as chelated radiometals or Al[^{18}F]$^{2+}$).[21, 24] The preparation of the nonradioactive fluorobenzoylated

peptides **5-8** by the reaction of nonradioactive SFB in DMF with peptides **1-4** in presence of DIPEA as base proceeded at room temperature in near quantitative yields as analyzed by HPLC (Table 1). The peptides were obtained in >95% purity after semi-preparative HPLC purification and were characterized by MALD-TOF and LC-MS.

Table 1. Structures of synthesized GnRH precursor peptides **1-4** and fluorobenzoyl labeled GnRH peptides **5-8** and purity determined by HPLC.



peptide	R_1	R_2	purity (%)
1	NH_2		98.4
2			95.0
3	NH_2		>99.0
.4			97.0
5			95.0
6			94.0

7			>99.0
8			98.4

In vitro affinity. The GnRH receptor affinities of the fluorobenzoylated peptides **5-8** were determined using a commercially available membrane preparation of human GnRHR-expressing cells and membranes preparations of HEK293 cells expressing rat GnRHR with [^{125}I]triptorelin as the radioligand. Results are summarized in Table 2 and representative inhibition curves are shown in Figure 1. All peptides were highly potent with subnanomolar affinities for both rat and human GnRHR. Among the group evaluated, peptide **7** with structure characteristics similar to the commercial GnRH superagonists deslorelin and buserelin which are 50 to 100 times more active than native GnRH, was highly potent, with a K_i-value around 50 pM for both GnRHR species.[25,26]

Table 2. pK_i with SEM and K_i values obtained for peptides **5- 8** for rat and human GnRHR (n= 3 or 4).

	Human GnRHR		Rat GnRHR	
Peptide	pK_i (± SEM)	K_i (*n*M)	pK_i (± SEM)	K_i (*n*M)
5	10.0 (± 0.5)	0.10	10.0 (± 0.1)	0.10
6	9.7 (± 0.2)	0.20	9.8 (± 0.3)	0.16
7	10.5 (± 0.4)	0.03	10.3 (± 0.2)	0.05
8	9.9 (± 0.4)	0.12	9.7 (± 0.1)	0.20

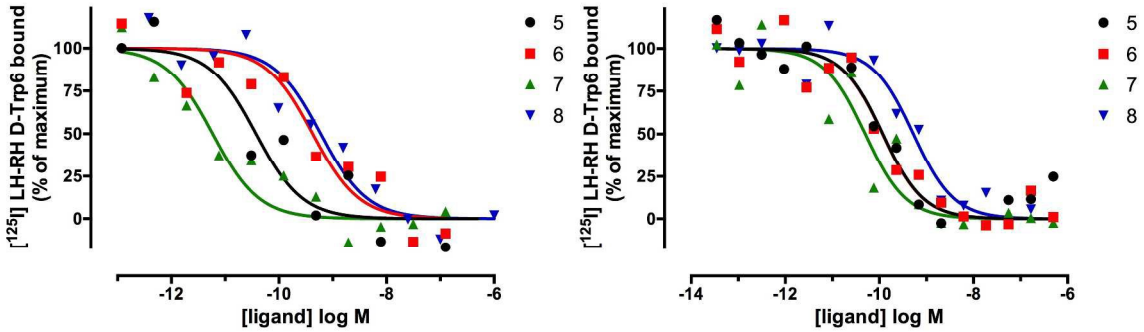


Figure 1. Representative inhibition-curves of the novel GnRH analogues in human (left) and rat (right) GnRHR expressing membrane preparations in competition with [^{125}I]triptorelin.

Radiosynthesis. Preparation of [^{18}F]**5-8** used the well-established [^{18}F]SFB route for the addition of the [^{18}F]FBA prosthetic group to peptides **1-4**. In typical experiments, a range of [^{18}F]SFB radioactivity was used (370 MBq – 11 GBq). Crude yields typically ranged from 50 to 60% using 1 mg of peptide precursor in 300 μL DMF at 40°C after 30 min as analyzed by analytical radio-HPLC. However, during purification of the ^{18}F-labeled peptides by semi-preparative HPLC an impurity eluted immediately before the product peak as a broad peak. Reducing collected fractions volume, increasing collection frequency and modification of the HPLC-gradient did not improve the radiochemical purity. It was observed that the impurity appeared to increase when higher radioactivity amounts of [^{18}F]SFB were used. In order to obtain a radiochemical purity (RPC) suitable for *in vivo* and *in vivo* studies (>95%) it was necessary to perform an additional HPLC purification using lower radioactivity. Typical specific activity for all peptide analogues was in the range 74 - 150 GBq/μmol at end of synthesis.

The lower purity is likely attributable to radiolysis as no degradation was observed for the

synthesis of the nonradioactive peptide standards. In order to test the hypothesis, pure peptide **5** was placed in a sealed conical vial containing 37 GBq of [^{18}F]fluoride in 1 mL saline. HPLC analysis after 15 min revealed a complete disappearance of the peak corresponding to **5** (t_R = 14.9 min), with two new major peaks eluting at 12.5 min and 25 min; by comparison, no degradation was observed for the matched control in saline. The experiment suggests that radioactivity has a profound effect on the stability of the ^{18}F-GnRH peptides, here represented by **5**.

Studies of radiolytic modifications in the GnRH-peptide sequence have shown that the amino acid residues tyrosine and tryptophan particularly prone to undergo radiolytic decomposition.[27] Identification of the decomposition products was not investigated further.

Log P determination. The log P values were measured in PBS pH 7.4 as the aqueous phase, corresponding to a physiological pH (Table 3). Interestingly, comparing of the four tracers [^{18}F]**5-8** showed that incorporation of the 6-aminohexanoyl amide spacer in [^{18}F]**6** and [^{18}F]**8** did not result in a substantial increase in log P values as compared to the unmodified versions [^{18}F]**5** and [^{18}F]**7**. At the same time, it was found that peptides with the desGly^{10}-NEt modified C-terminus ([^{18}F]**7** and [^{18}F]**8**) showed an increase in log P value compared to their full-length GnRH counterparts (Table 3). Overall, the four peptides had, as expected, log P values lower than found for CNS tracers with passive BBB diffusion (log P range 3-5).[28] However, the log P was in the same range as the iodinated GnRH peptide studied by Barrera and co-worker, measured to be 0.037, and if any active transport is involved in mediating the transport of GnRH peptides across of the BBB, log P-partition coefficients do have limited predictive value for brain uptake.[17]

Table 3. Measured Log P values for ^{18}F-peptides at pH 7.4 in PBS (n=4)

Compound	[^{18}F]**5**	[^{18}F]**6**	[^{18}F]**7**	[^{18}F]**8**
Log P value	-0.753 (±0.01)	-0.045 (±0.01)	0.013 (±0.02)	0.257 (±0.01)

Serum stability. Stability of the ^{18}F-peptides in rat serum was investigated after 1 and 2 h. In accordance with previously published literature, the 6-aminohexanoyl amide modified peptides [^{18}F]**6** and [^{18}F]**8** exhibited improved stability, with only a 5% reduction in RCP after 2 h as compared to peptides [^{18}F]**5** and [^{18}F]**7** with a 30% decline after 2 h.[20]

In vivo stability. In order to investigate the *in vivo* stability in rats [^{18}F]**7** was injected and blood was collected and analyzed by radio-HPLC. Due to the fast clearance of the ^{18}F-peptides from systemic circulation, blood collected later than 10 min post injection did not contain sufficient radioactivity for radio-HPLC analysis. Gratifyingly, as shown in Figure 2, after 10 min *in vivo*, most of [^{18}F]**7** in blood remained intact (> 94%). Urine was collected after 2.5 h following the PET/CT scans, as a part of the biodistribution studies. As expected, the fraction of intact ^{18}F-peptides was drastically reduced in urine, with only 10-17% of the total collected radioactivity associated with intact ^{18}F-peptides. These data agree well with those found by Heinrich *et a*l. for the closely related GnRH peptide buserelin.[29] The extensive degradation observed in urine is likely a result of further proteolytic breakdown in the brush border membrane of the kidney tubuli.[30]

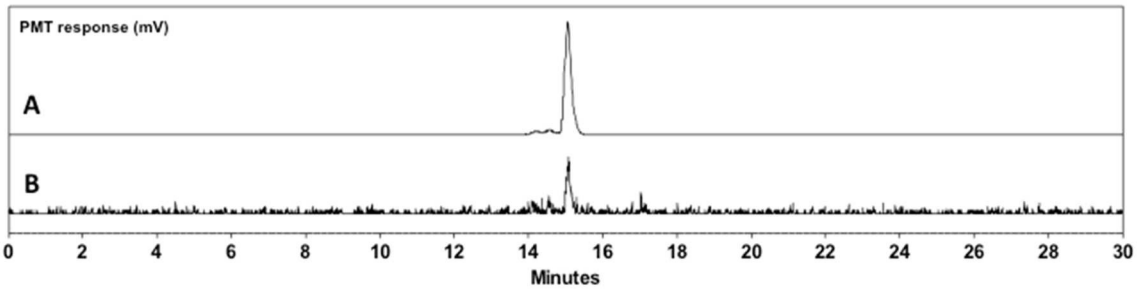


Figure 2. Radio-HPLC chromatogram (A): Formulated [^{18}F]**7** prior to injection into rat. The major peak at t_R = 15.1 min is intact [^{18}F]**7**. (B): [^{18}F]**7** from rat blood collected 10 min after injection. The major peak also elutes at t_R = 15.1 min.

PET Imaging and biodistribution. PET images of a rat after injection of [^{18}F]**7** are shown in Figure 3. No brain uptake could be observed by PET during the time frame of the experiments (2 h). This was also confirmed by the biodistribution study where <0.005% ID/g [^{18}F]**7** were found in the brain at 2 h post injection. A similar lack of uptake was also observed for the other ^{18}F-GnRH peptides [^{18}F]**5**, [^{18}F]**6** and [^{18}F]**8**. Thus, taken together, our observations are more closely aligned with those by Verheugen *et al.* who concluded that the BBB is impermeable to GnRH and do not agree well with the findings of Barrera *et al*. that suggested an active bidirectional transport.[17,19] In this context it is important to note that the methodology used by Barrera *et al*. involved carotid occlusion that may have confounded their results by making the BBB temporally more permeable, or if the measured radioactivity had in fact crossed the BBB or was merely bound to the endothelium. Given the high specific activities of the tracers used in our study, the mass of injected peptide was around 400 pmol/injection, assuming a specific activity of 75 GBq/μmol compared to 1.85 GBq/μmol obtained by Barrera *et al*.

gives us confidence that a self-blocking effect due to excess carrier can be ruled out for our *in vivo* studies.

A more recent study conducted by Caraty *et al*. showed that i.v. administered GnRH possessed the ability to penetrate the cerebroventricular system; however, it appears to occur predominately and possibly exclusively, through the median eminence region. Notably, high doses of GnRH (1 mg, 845 nmol) were required to produce measurable and physiological concentrations in the CSF (40 pg/mL) in ewes; these doses were 2100 higher than the amounts used in our tracer studies, and therefore these results appear to be in accordance with our findings that any possible transport of GnRH across the BBB under normal physiological conditions can be expected to occur at very low levels.[18] Despite the very high sensitivity of PET imaging such a possible active transport mechanism, if it exists, may go undetected by our ^{18}F-peptides because they, like other GnRH peptides, clear quickly from the blood, thereby severely limiting the window of time available for interaction with the blood-brain barrier.

Interestingly, a region at the base of the brain showed relatively high tracer uptake (Figures 3 and 4). This location aligns with the pituitary gland, located outside the BBB, and is the principal target for GnRH agonist treatment. The reported receptor density of GnRHR in rat pituitary tissue ranges from 11 to 140 fmol receptor/mg tissue as determined by using similar ^{125}I-iodinated GnRH peptide analogues.[31-33] To determine that the tracer uptake in the pituitary observed in our *in vivo* study was due to GnRHR specific binding, a blocking study with the GnRH agonist buserelin (300 μg/kg) was performed for [^{18}F]**7**. This blocking study demonstrated a significant reduction (five-fold) in pituitary uptake 1 h p.i. when compared to injection of [^{18}F]**7** alone, indicating

GnRHR-mediated pituitary binding of the ^{18}F-peptide in *vivo* and demonstrating the ^{18}F-GnRH peptide's ability to successfully image low density GnRHR tissues *in vivo*. Time-activity curves showing uptake in pituitary and brain in the absence and in presence of buserelin are shown in Figure 4. The curves show a fast binding of the ^{18}F-peptide [^{18}F]**7** to the pituitary that peaks around 10 min and remains relatively high for approximately 1 hour after injection. During the same time frame, radioactivity clears well from surrounding non-target tissues, thereby giving good target-to-background ratios of about 5/1 to 10/1 during this period.

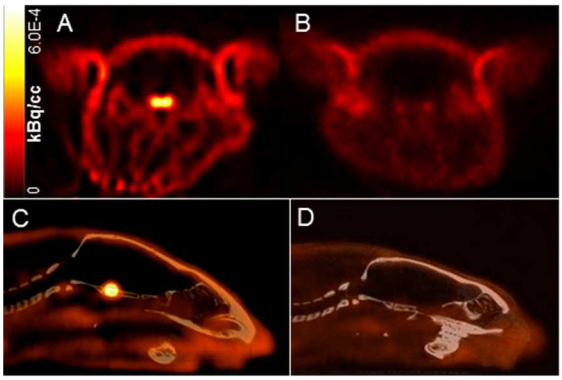


Figure 3. *In vivo* imaging. (A) and (B): Coronal small animal PET images (summed from 30 to 120 min) in the plane of the pituitary gland of a rat injected with 26 MBq [^{18}F]**7**. (A): Administration of [^{18}F]**7** only; (B): Administration of [^{18}F]**7** 10 min after administration of 100 μg buserelin. (C) and (D): co-registered sagittal PET/CT images as above in the plane of the pituitary gland.

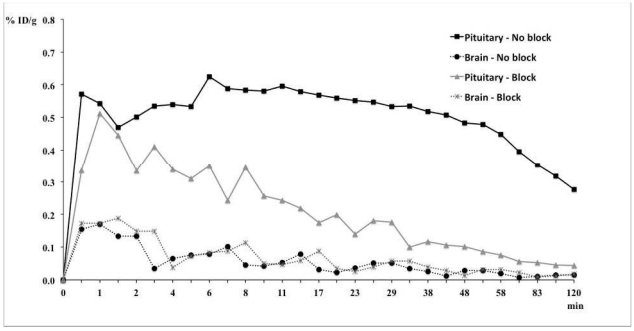


Figure 4. Time–activity curves for [^{18}F]**7** in brain and pituitary with (block) and without (no block) administration of 100 μg of buserelin ten minutes prior to [^{18}F]**7**.

Combined biodistribution data in naive male rats for ^{18}F-peptides **5-8** (left column, no block) and for [^{18}F]**7** ten minutes after administration of buserelin **(**100 μg) (right column, block) are shown in Table 4 120 min p.i. These additional biodistribution data obtained for the tracers at the 2.5 h time-point further revealed that all peptide tracers cleared rapidly from the blood (0.05-0.09% ID/g) with low retention in bone (0.03-0.08% ID/g) and muscle (0.01-0.06% ID/g). By comparison, kidney and intestinal uptake were relative high at 0.4-1.0 % and 0.5-3.0% ID/g, respectively, indicating clearing via the renal and hepatobiliary pathways. The renal pathway was dominating, with 30-60% ID/g of the radioactivity recovered from urine. Overall, the data show that all four analogs evaluated behaved very similarly *in vivo*; in comparison to the peptide ^{111}In-DOTA-Ahx-(D-Lys^{6}-GnRH) at 2 h[34], a radiolabeled GnRH peptide that also includes a D-Lys^{6} and had previously been investigated for breast cancer imaging, the uptake in non-target organs were comparable, except for the kidneys, the uptake for the ^{18}F-GnRH peptides reported in the present study was 6 to 8-fold lower than that of the DOTA-GnRH peptide (8.88% ID/g at 2 h).

Uptake in organs other than the pituitary remained similar in absence and presence of buserelin suggesting a low GnRHR expression in these. Hence, it would be interesting to examine the utility of these novel ^{18}F-peptides in tumor models expressing GnRHR. A full biodistribution study was not performed, as the primary goal of the current investigations was to study the potential CNS distribution of the ^{18}F-peptides.

Table 4. Combined biodistribution of ^{18}F-peptides in male rats 120 min p.i.

Organ	Percent Injected Dose/gram [% ID/g]	
	No block[a] (± SD)	Block[b]
blood	0.07 ± 0.02	0.05
bone	0.05 ± 0.03	0.03
brain	0.01 ± 0.00	0.00
heart	0.05 ± 0.02	0.04
intestines	2.05 ± 0.61	0.53
kidneys	1.14 ± 0.68	0.06
lung	0.12 ± 0.06	0.12
liver	0.44 ± 0.29	0.43
muscle	0.03 ± 0.08	0.02
skin	0.21 ± 0.05	0.13
spleen	0.13 ± 0.08	0.27
pituitary[c]	0.28	0.04
	Uptake Ratio of Pituitary/Normal tissue	
Pituitary/blood	4	0.8
Pituitary/muscle	9.3	2

[a] Data are mean of [^{18}F]**5**, [^{18}F]**6,** [^{18}F]**7** and [^{18}F]**8** where n = 1 for each ^{18}F-peptide

[b] Biodistribution data of [^{18}F]**7** injected ten minutes after buserelin (n = 1) for each ^{18}F-peptide

[c] Data from time-activity-curve of [^{18}F]**7** at 2 h were converted to % ID/g using the calibration factor obtained from the calibration cylinder

Conclusion

The purpose of this work was to perform an initial evaluation of the brain uptake and potential specific binding of GnRH peptides labeled with fluorine-18 based on the previously suggested ability of GnRH peptides to cross into the brain. Despite the very high sensitivity of PET imaging, the *in vivo* experiments revealed no measurable brain uptake of the ^{18}F-peptides. Modification of the peptides by introducing six-carbon spacer at Lys^{6} or a C-terminal Pro^{9}-ethylamide in order to increase hydrophobicity did not translate in any observable effect on brain uptake.

Despite this lack of brain uptake, the ^{18}F-labeled peptides did show very high affinity towards rat and human GnRH receptor *in vitro*; and in particular the ability for the

radiolabeled peptide [^{18}F]FBA-D-Lys^{6}, Pro^{9}-NEt-GnRH (**7**) to image tissue expressing low levels of GnRH receptor was demonstrated *in vivo* in the pituitary gland. The combination of high GnRH receptor affinity, good *in vivo* stability, and a favorable pharmacokinetic profile of the ^{18}F-peptides reported here warrants further studies, such as for imaging and therapy of GnRH-responsive tumor models.

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Notes

The authors declare no competing financial interest.

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