

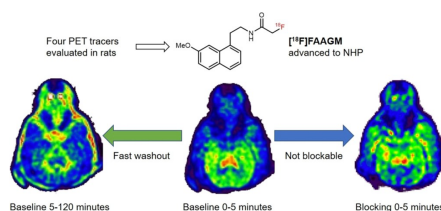
This is the postprint (accepted) version of the final paper, published afterwards, here the link:

<https://doi.org/10.1021/acschemneuro.1c00678>

We thank ACS for the opportunity.

## Radiosynthesis and *In Vivo* Evaluation of Four Positron Emission Tomography Tracer Candidates for Imaging of Melatonin Receptors

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**Abstract:** Melatonin is a neurohormone that modulates several physiological functions in mammals through the activation of melatonin receptor type 1 and 2 (MT<sub>1</sub> and MT<sub>2</sub>). The melatonergic system is an emerging therapeutic target for new pharmacological interventions in the treatment of sleep and mood disorders; thus, imaging tools to further investigate its role in the brain are highly sought-after. We aimed to develop selective radiotracers for *in vivo* imaging of both MT<sub>1</sub> and MT<sub>2</sub> by positron emission tomography (PET). We identified four previously reported MT ligands with picomolar affinities to the target based on different scaffolds which were also amenable for radiolabeling with either carbon-11 or fluorine-18. [<sup>11</sup>C]UCM765, [<sup>11</sup>C]UCM1014, [<sup>18</sup>F]3-fluoroagomelatine ([<sup>18</sup>F]3FAGM), and [<sup>18</sup>F]-fluoroacetamidoagomelatine ([<sup>18</sup>F]FAAGM) have been synthesized in high radiochemical purity and evaluated in wild-type rats. All four tracers showed moderate to high brain permeability in rats with maximum standardized uptake values (SUV<sub>max</sub> of 2.53, 1.75, 3.25, and 4.47, respectively) achieved 1–2 min after tracer administration, followed by a rapid washout from the brain. Several melatonin ligands failed to block the binding of any of the PET tracer candidates, while in some cases, homologous blocking surprisingly resulted in increased brain retention. Two <sup>18</sup>F-labeled agomelatine derivatives were brought forward to PET scans in non-human primates and autoradiography on human brain tissues. No specific binding has been detected in blocking studies. To further investigate pharmacokinetic properties of the putative tracers, microsomal stability, plasma protein binding, log *D*, and membrane bidirectional permeability assays have been conducted. Based on the results, we conclude that the fast first pass metabolism by the enzymes in liver microsomes is the likely reason of the failure of our PET tracer candidates. Nevertheless, we showed that PET imaging can serve as a valuable tool to investigate the brain permeability of new therapeutic compounds targeting the melatonergic system.

**KEYWORDS:** melatonin, agomelatine, melatonin receptors, positron emission tomography, PET, carbon-11, fluorine-18, [<sup>11</sup>C]UCM765, [<sup>11</sup>C]UCM1014, [<sup>18</sup>F]3FAGM, [<sup>18</sup>F]FAAGM

### INTRODUCTION

Melatonin (*N*-acetyl-5-methoxytryptamine, MLT) is a sleep-inducing hormone secreted in the central nervous system (CNS) of mammals mainly from the pineal gland during the dark phase of the light/dark cycle.<sup>1</sup> MLT regulates circadian rhythms *via* activation of two types of G protein-coupled receptors of the G<sub>i/o</sub> type, known as melatonin type 1 and 2 (MT<sub>1</sub> and MT<sub>2</sub>) receptors.<sup>2</sup> Each receptor is located in specific areas of the brain<sup>3,4</sup> and has a distinct function in the regulation of neurophysiological functions.<sup>5</sup> Besides their role in sleep, circadian rhythm, and pain, a growing body of literature suggests that aberrant expression of melatonin receptors is implicated in the pathogenesis of neuropsychiatric disorders (anxiety and depression)<sup>6</sup> and neurodegenerative diseases, including Alzheimer's disease,<sup>7,8</sup> Parkinson's disease,<sup>9</sup> and Huntington's disease.<sup>10</sup> Therefore, the melatonergic system has recently emerged as a promising therapeutic target.<sup>6,11,12</sup> Synthetic MLT has been extensively used as an over-the-counter medication for the treatment of insomnia.<sup>13</sup>

In the past 2 decades, several non-selective MT<sub>1</sub>/MT<sub>2</sub> agonists with longer biological half-lives have been approved for the treatment of sleep disorders, including ramelteon, tasimelteon, and agomelatine.<sup>14</sup> The latter is also approved in Europe as an atypical antidepressant for treatment of major depressive disorder<sup>15</sup> and showed efficacy in depression associated with neurodegenerative diseases<sup>16</sup> and other comorbid conditions.<sup>17</sup> Accordingly, there is an emerging demand for the development of ligands selective to distinct melatonin receptor types. Several reviews on MT<sub>1</sub>- and MT<sub>2</sub>-selective synthetic ligands have been recently published.<sup>18,19</sup>

Despite these fast pharmacological advances, neuroanatomical mapping of melatonin receptor distribution in mammalian brains remains incomplete, particularly due to the dearth of reliable specific antibodies<sup>20</sup> as well as MT<sub>1</sub>- and MT<sub>2</sub>-selective radioligands.<sup>21</sup> Autoradiography studies using 2-[<sup>125</sup>I]-iodomelatonin<sup>22</sup> (Figure 1) greatly contributed to the pharmacological characterization and localization of MT receptors in the 80s and 90s.<sup>23,24</sup> While the results were inconsistent, most publications reported expression of melatonin binding sites with  $B_{\text{max}}$  values in the range of 3–50 fmol/mg protein in small hypothalamic nuclei, including the suprachiasmatic nucleus (SCN), median eminence (ME), and pars tuberalis in the pituitary gland, and in the cerebellar cortex across several mammalian species.<sup>25–29</sup>

Although 2-[<sup>125</sup>I]iodomelatonin binds to melatonin binding sites with low picomolar affinity (reported  $K_d$ 's in the range of 10–80 pM),<sup>25–29</sup> it does not differentiate between MT<sub>1</sub> and MT<sub>2</sub> and it has been suggested that dimerization of melatonin receptors can significantly reduce the number of available binding sites.<sup>30</sup> Recently, immunohistochemistry studies<sup>3</sup> and the knock-in genetic strategy replacing MT<sub>1</sub> or MT<sub>2</sub> coding sequences with a LacZ reporter<sup>4</sup> have reported that MT<sub>1</sub> and MT<sub>2</sub> receptors are localized in distinct regions; for example, MT<sub>2</sub> were found mostly in the thalamic regions, the hippocampus and brainstem. The Human Protein Atlas database reports basal ganglia (BG) as the only region of MT<sub>2</sub> localization in the human brain on the RNA level (MTNR1B gene), but on a protein level, it is found in the cortex, hippocampus, caudate, and cerebellum; however, in pigs, the highest expression of MT<sub>2</sub> was found in the midbrain, pons/medulla, and corpus callosum.<sup>31</sup> According to the same atlas, the MT<sub>1</sub>-encoding gene MTNR1A is instead localized mostly in the human cerebellum, while in pigs, it is localized primarily in the hypothalamus and pituitary gland.<sup>32</sup> The Allen Brain Atlas reports MT<sub>2</sub> in the mouse brain only in negligible amounts in the cerebellum and does not report any expression of MT<sub>1</sub>.<sup>33</sup> In addition, neither autoradiographic nor immunohistochemistry methods allow for *in vivo* imaging of MT<sub>1</sub> and MT<sub>2</sub>. Accordingly, there is a great necessity to develop imaging tools with high selectivity and specificity to MT receptors for the visualization of their localization, concentration, and function in different brain regions in living organisms as well as for the development of new drugs targeting the melatonergic system. Positron emission tomography (PET) is a powerful tool in neuroimaging studies for *in vivo* imaging of receptor expression. To date, none of the radioligands labeled with either carbon-11 or fluorine-18 have been validated as potential PET tracers for melatonin receptor imaging despite significant efforts in the early days of PET imaging developments (Figure 1). Thus, [<sup>11</sup>C]MLT showed no specific binding in the brain despite fast permeation of the blood brain barrier (BBB),<sup>34</sup> most likely due to competition with endogenous MLT resulting in poor molar activity *in vivo*. PET imaging using either 4- or 6-[<sup>18</sup>F]fluoromelatonin synthesized *via* electrophilic <sup>18</sup>F-fluorination of MLT has not been reported;<sup>35</sup> however, it can be assumed that at the obtained molar activities of below 0.01 GBq/μmol, no specific binding would be observed. 2-Iodo-[<sup>11</sup>C]melatonin was synthesized with a moderate molar activity of 11–22 GBq/μmol and advanced to imaging in human subjects, but no specific binding was detected as the tracer was rapidly cleared from the brain.<sup>36</sup> The isotopologue 2-[<sup>123</sup>I]iodomelatonin was suggested as an SPECT tracer but also did not reveal any specificity, presumably due to rapid *in vivo* deiodination.<sup>37</sup> Most recently, [<sup>11</sup>C]ramelteon was labeled *via* <sup>11</sup>C-acylation in high decay-corrected radiochemical yield (RCY) and modest molar activity, but no biological evaluation of this PET tracer was reported.<sup>38</sup>

To the best of our knowledge, no attempt to evaluate MT-selective PET tracers was reported since the 1990s. Recent developments in pharmacology yielded numerous novel, more potent, and selective MT ligands, while modern PET scanners offer significantly higher sensitivity and resolution compared to the technology used in earlier studies. These advances prompted us to reinvigorate the efforts to develop MT-specific PET tracers. We identified four previously reported ligands with  $K_i$  values below 100 pM to at least one of the MT receptor types and amenable for radiolabeling with one of the most common PET isotopes, carbon-11 or fluorine-18 (Figure 2). UCM765<sup>39</sup> is a selective MT<sub>2</sub> partial agonist which has been reported to promote non-rapid eye movement sleep maintenance<sup>40</sup> and has anxiolytic<sup>41</sup> and antinociceptive properties<sup>42</sup> in experimental animals. UCM1014<sup>43</sup> is a more recently developed full agonist with improved affinity toward MT<sub>2</sub> and a 17000-fold selectivity over MT<sub>1</sub>. Both these ligands contain methoxy groups, making them amenable for radio-labeling *via* <sup>11</sup>C-methylation for subsequent evaluation as MT<sub>2</sub>-selective PET tracers. Two isomeric fluoroagomelatine derivatives, *N*-(2-(3-fluoro-7-methoxynaphthalen-1-yl)ethyl)-acetamide (3-fluoroagomelatine) and 2-fluoro-*N*-(2-(7-methoxynaphthalen-1-yl)ethyl)acetamide (fluoroacetamidoagomelatine), respectively termed 3FAGM and FAAGM hereafter, were reported as part of a structure–activity relationship study aiming to improve the affinity and metabolic stability of the parent agomelatine.<sup>44</sup> Both compounds are among the most potent ligands, with nearly equipotent picomolar affinities to MT<sub>1</sub> and MT<sub>2</sub>. Hence, their <sup>18</sup>F-fluorinated analogues could serve as PET tracers for non-selective imaging of MT receptors. Besides high affinities to the target, all four molecules displayed good to excellent calculated CNS multiparameter optimization (MPO) scores (Table 1). The CNS MPO score was developed by Wager *et al.*<sup>45,46</sup> as a predictive tool for brain permeability of drug candidates by assigning a score between 0 and 6.0 based on six physicochemical properties. CNS MPO has been widely adopted in PET tracer development for brain imaging with a score of 4.0 as a threshold to select the candidates with a higher probability of brain entry. All four ligands satisfy this requirement. In this study, we report the radiosyntheses and *in vivo* studies of the brain permeability, CNS regional distribution, and washout rate for these four ligands in rats and non-human

primates and their evaluation as PET tracer candidates for the imaging of melatonin receptors.

## Results and Discussion

**Chemistry.** N-(2-((3-Methoxyphenyl)(phenyl)amino)-ethyl)acetamide (UCM765),<sup>39</sup> N-((1-benzyl-5-methoxy-1,2,3,4-tetrahydroquinolin-2-yl)methyl)propionamide (UCM1014),<sup>43</sup> N-(2-(3-fluoro-7-methoxynaphthalen-1-yl)-ethyl)acetamide (3-fluoroagomelatine, 3FAGM), and 2-fluoro-N-(2-(7-methoxynaphthalen-1-yl)ethyl)acetamide (fluoroagomelatine, FAAGM)<sup>44</sup> have been synthesized according to the previously reported procedures and used as non-radioactive standards for the optimization of conditions for both preparative and analytical high-performance liquid chromatography (HPLC). The former two compounds were treated with boron tribromide to yield desmethyl precursors dmUCM765 and dmUCM1014 for subsequent radiosyntheses of [<sup>11</sup>C]UCM765 and [<sup>11</sup>C]UCM1014, respectively, via <sup>11</sup>C-methylation. The boronic acid pinacolate precursor N-(2-(7-methoxy-3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)naphthalen-1-yl)ethyl)acetamide (3-BpinAGM) for the radiosynthesis of [<sup>18</sup>F]3FAGM was synthesized via Miyaura borylation from the previously reported N-(2-(3-bromo-7-methoxynaphthalen-1-yl)ethyl)acetamide (3-bromoagomelatine, 3-BrAGM). 2-Bromo-N-(2-(7-methoxynaphthalen-1-yl)ethyl)acetamide (bromoacetamidoagomelatine, BrAAGM), the precursor for the radiosynthesis of [<sup>18</sup>F]FAAGM, was synthesized by acylation of 2-(7-methoxynaphthalen-1-yl)ethan-1-amine with bromoacetyl bromide. Syntheses of all four precursors starting from either commercially available or previously reported compounds are summarized in Scheme 1. **Radiochemistry.** [<sup>11</sup>C]UCM765 and [<sup>11</sup>C]UCM1014 were synthesized from the corresponding desmethyl precursors dmUCM765 and dmUCM1014, respectively, and [<sup>11</sup>C]CH<sub>3</sub>I in DMSO in the presence of sodium hydroxide at 90 °C (Scheme 2). Both tracers were produced in moderate isolated decay-corrected RCYs of 17–22% (n = 4) and 18% (n = 1), respectively, with radiochemical purities (RCPs) of >99% and molar activities (Am) of 372 ± 60 GBq/μmol in 18–22 min. [<sup>18</sup>F]3FAGM was synthesized via late-stage copper-catalyzed <sup>18</sup>F-fluorination of the corresponding boronic acid pinacolate precursor 3BpinAGM in a 2:1 DMA/1-butanol mixture at 130 °C with a decay-corrected RCY of 20.3 ± 6.6%, an RCP of >99%, and Am of 108.7 ± 47.5 GBq/μmol in 75 min (n = 2) (Scheme 3, left). [<sup>18</sup>F]FAAGM was synthesized via S<sub>N</sub>2 substitution of the bromide in the corresponding bromoacetamide precursor BrAAGM at 100 °C in acetonitrile for 15 min with a decay-corrected RCY of 10.7 ± 6.6%, an RCP of >99%, and Am of 281.9 ± 147.5 GBq/μmol in 55 min (n = 2).

CNS MPO score was developed by Wager et al.<sup>45,46</sup> as a predictive tool for brain permeability of drug candidates by assigning a score between 0 and 6.0 based on six physicochemical properties. CNS MPO has been widely adopted in PET tracer development for brain imaging with a score of 4.0 as a threshold to select the candidates with a higher probability of brain entry. All four ligands satisfy this requirement. In this study, we report the radiosyntheses and in vivo studies of the brain permeability, CNS regional distribution, and washout rate for these four ligands in rats and non-human primates and their evaluation as PET tracer candidates for the imaging of melatonin receptors. (Scheme 3, right).

All tracers were purified by preparative HPLC and reformulated in dilute ethanol solution in saline suitable for intravenous injection in animals. Radiochemical and chemical purities, ligand identities, and molar activities were determined by analytical HPLC prior to tracer injection into animals.

**PET Imaging in Rodents.** [<sup>11</sup>C]UCM765 and [<sup>11</sup>C]UCM1014 were evaluated first as PET tracer candidates for selective imaging of the MT<sub>2</sub> receptor in wild-type Sprague–Dawley rats to determine their brain permeation, regional distribution, and clearance rate. Baseline [<sup>11</sup>C]UCM765 scans revealed that the tracer rapidly crosses the BBB, with a whole brain SUV<sub>max</sub> of 2.53 ± 0.45 (n = 4) achieved 30 s post injection (Figure 3). The tracer shows nearly homogeneous distribution in all analyzed brain regions despite the minor differences in the initial uptake in the following order: thalamus (SUV<sub>max</sub> = 3.00 ± 0.42), hippocampus (SUV<sub>max</sub> = 2.62 ± 0.40), cerebellum (SUV<sub>max</sub> = 2.53 ± 0.50), hypothalamus (SUV<sub>max</sub> = 2.51 ± 0.49), and cortex (SUV<sub>max</sub> = 2.34 ± 0.49). The minor differences in the tracer uptake are erased within 10 min as the tracer underwent rapid washout from all analyzed brain regions with the standardized uptake values (SUVs) decreasing below 0.5 within 20 min after injection. Consistent with its lower CNS MPO score, [<sup>11</sup>C]UCM1014 showed a poorer brain uptake at the baseline scan (global SUV<sub>max</sub> = 1.75, n = 1) compared to [<sup>11</sup>C]UCM765 and, therefore, was not evaluated in further blocking studies (Figure 4).

Next, we performed a series of *in vivo* blocking experiments by injecting selective MT<sub>2</sub> antagonist 4P-PDOT (10 mg/kg, s.c., n = 1) shown to be competitive with UCM765,<sup>41</sup> synthetic MLT (150 mg/kg, s.c., n = 1), or non-radioactive UCM765 (20 mg/kg, i.v., n = 1) prior to the injection of [<sup>11</sup>C]UCM765 (Figure 5). No significant reduction of the brain uptake has been observed with 4P-PDOT, whereas administration of MLT resulted in a slight reduction in tracer brain uptake in the first 5 min of the scan, which can be attributed to restricted cerebral blood flow due to the excessive dose of the administered MLT (150 mg/kg).<sup>47</sup> A homologous blocking experiment surprisingly resulted in higher apparent tracer brain uptake compared to the baseline scan. We hypothesize that UCM765 is a substrate of metabolic enzymes and saturating those enzymes with a non-radioactive compound results in a longer apparent half-life of [<sup>11</sup>C]UCM765, or a competitive binding occurs at the extracerebral sites of MT<sub>2</sub> expression. Accordingly, the tracer remains longer in the blood circulation and increases the apparent radioactivity signal from the brain. This

observation is in accordance with a previously reported short half-life of the parent compound *in vitro* in the presence of rat microsomes ( $t_{1/2} = 1.7$  min).<sup>48</sup>

We then evaluated two <sup>18</sup>F-fluorinated agomelatine derivatives, [<sup>18</sup>F]3FAGM and [<sup>18</sup>F]FAAGM, as PET tracer candidates for non-selective imaging of MT<sub>1</sub> and MT<sub>2</sub> in wild-type Sprague–Dawley rats. Both tracers showed excellent brain permeability within 1 min after tail-vein injection with a global SUV<sub>max</sub> of  $3.25 \pm 0.35$  ( $n = 4$ ) for [<sup>18</sup>F]3FAGM (Figure 6) and  $4.47 \pm 0.22$  ( $n = 2$ ) for [<sup>18</sup>F]FAAGM (Figure 7).

Similar to baseline [<sup>11</sup>C]UCM765 scans, both tracers showed nearly homogenous distribution in all analyzed brain regions, despite the minor differences in the initial uptake with the highest uptake in the thalamus (SUV<sub>Th</sub> =  $3.89 \pm 0.51$  and  $4.67 \pm 0.18$  for [<sup>18</sup>F]3FAGM and [<sup>18</sup>F]FAAGM, respectively) and the lowest uptake in the cortex (SUV<sub>Ctx</sub> =  $2.91 \pm 0.33$  and  $3.93 \pm 0.48$  for [<sup>18</sup>F]3FAGM and [<sup>18</sup>F]FAAGM, respectively). Also similar to [<sup>11</sup>C]UCM765 scans, uptake of both [<sup>18</sup>F]3FAGM and [<sup>18</sup>F]FAAGM was followed by a rapid washout from all analyzed brain regions.

Blocking experiments with either agomelatine (10 mg/kg, s.c.,  $n = 2$  for [<sup>18</sup>F]3FAGM,  $n = 2$  for [<sup>18</sup>F]FAAGM) or MLT (150 mg/kg, s.c.,  $n = 2$  for [<sup>18</sup>F]3FAGM) does not affect SUV<sub>max</sub> but leads to higher retention of both tracers in the later frames of the scans in rats (Figures 8 and 9). However, neither agomelatine- nor MLT-induced increase in the global retention of [<sup>18</sup>F]3FAGM and [<sup>18</sup>F]FAAGM was statistically significant ( $0.05 < p < 0.1$ ). At the regional level, the uptake of [<sup>18</sup>F]3FAGM was significantly higher in the cortex, cerebellum, thalamus, and hippocampus following agomelatine administration. Similarly, a significant agomelatine-induced increase in [<sup>18</sup>F]FAAGM uptake was observed in the thalamus and hippocampus. These results could be attributed to the fact that [<sup>18</sup>F]3FAGM and [<sup>18</sup>F]FAAGM are substrates to hepatic microsomal enzymes, where the latter were saturated by agomelatine, which had led to higher regional uptake of these two tracers. MLT, on the other hand, did not show statistically significant changes in [<sup>18</sup>F]3FAGM retention, neither globally nor regionally.

**PET Imaging in Non-human Primates.** To further evaluate these tracers in higher species, both [<sup>18</sup>F]3FAGM and [<sup>18</sup>F]FAAGM were advanced to PET studies in non-human primates. In good correlation with scans in rodents, [<sup>18</sup>F]FAAGM showed higher brain permeability (SUV<sub>max</sub> =  $3.39 \pm 0.06$ ,  $n = 2$ ) than [<sup>18</sup>F]3FAGM (SUV<sub>max</sub> =  $2.37$ ,  $n = 1$ ) in macaques and, therefore, was brought forward to blocking studies using agomelatine (i.v. 4 mg/kg,  $n = 2$ ). Using cerebellum white matter as a reference region devoid of melatonin receptor expression,<sup>28</sup> we analyzed Logan distribution volume ratios (DVRs) in all other brain regions reported to express either MT<sub>1</sub> or MT<sub>2</sub> including the pituitary gland, hypothalamus, thalamus, cerebellum gray matter, and BG. The slope of the Logan plot was estimated using the time period from 5 to 120 min. At the baseline, DVR<sub>5–120</sub> in all regions were below 1 (pituitary gland:  $0.78 \pm 0.07$ , hypothalamus:  $0.76 \pm 0.10$ , thalamus:  $0.97 \pm 0.05$ , cerebellum gray matter:  $0.79 \pm 0.01$ , and BG:  $0.74 \pm 0.04$ ), suggesting a preferential non-specific binding of the tracer to a white matter (Figure 10). In addition, no statistically significant blocking has been detected in any of the analyzed regions ( $\Delta$ DVR<sub>5–120</sub> as follows: pituitary gland:  $-6.9 \pm 24.2\%$ , hypothalamus:  $-2.3 \pm 3.0\%$ , thalamus:  $2.4 \pm 4.2\%$ , cerebellum gray matter:  $-2.7 \pm 2.7\%$ , and BG:  $1.6 \pm 1.2\%$ ). **Autoradiography.** Finally, we performed a series of autoradiography experiments on rat and human brain slices using one tracer from each class of lead molecules: [<sup>11</sup>C]- UCM765 and [<sup>18</sup>F]FAAGM. No preferential binding of [<sup>11</sup>C]UCM765 in the brain regions reported to express MT<sub>2</sub> was observed. Furthermore, none of 4P-PDOT, MLT, and UCM765 (10  $\mu$ M) reduced the tracer binding in any of the analyzed brain regions (Figures S5–S7). On the other hand, baseline autoradiography with [<sup>18</sup>F]FAAGM on human brain tissues showed excessive binding in regions rich with white matter, such as cerebellum white matter, consistent with the *in vivo* PET data. Coincubation with either melatonin (10  $\mu$ M) or agomelatine (10  $\mu$ M) did not reduce the tracer binding in any of the analyzed brain regions, including the midbrain, hippocampus, pituitary gland, and cerebellum (Figure S8). As an example, the ratio of tracer accumulation in the cerebellum gray matter to cerebellum white matter was 0.29, while the blocking in the cerebellum gray matter was not statistically significant with both melatonin ( $5.2 \pm 9.8\%$ ) and agomelatine ( $4.7 \pm 6.7\%$ ).

**In Vitro Biological Assays.** In order to further investigate the failure of our candidates to specifically detect the expression of melatonin receptors both in PET and in autoradiography studies, we performed a series of *in vitro* biological assays, including measurements of log *D*, microsomal stability (rat), plasma protein binding (rat), and bidirectional permeability assessment of P-gp efflux susceptibility (MDCK-mdr1 transfected line). The results of these assays are summarized in Table 2.

The data show that both compounds have suitable lipophilicity (log *D*<sub>7.4</sub>  $\sim 2.6$ – $3.0$ ) and plasma free fraction ( $f_p \sim 5$ – $10\%$ ) and neither is subjected to P-gp efflux. However, both FAAGM and 3FAGM undergo a rapid metabolism in the presence of rat liver microsomes as both compounds show a half-life below 10 min and a microsomal clearance of  $\sim 80\%$ . This suggests a rapid *in vivo* metabolism of our fluorinated agomelatine tracer candidates, which explains its fast washout from the brain and increased brain exposure upon coinjection with agomelatine as they are likely the substrates of the same drug-metabolizing enzymes. Preferential binding to white matter in autoradiography experiments can be explained by the relatively high, albeit acceptable, lipophilicity of both tracers. We therefore conclude that the agomelatine scaffold is likely not suitable for future PET tracer development efforts for melatonin receptors due to its susceptibility to the *in vivo* metabolism.

## Conclusions

We developed the radiosynthesis of four different PET tracer candidates for the imaging of melatonin receptors and evaluated their brain permeability, regional distribution, washout rate, and binding specificity in rats. All tracers exhibit moderate to high and rapid brain entry in the baseline experiments with almost homogeneous distribution in all analyzed brain regions. Administration of several structurally different melatonin receptor ligands increased brain uptake of all of the investigated tracer candidates. The most brain-permeable tracer [ $^{18}\text{F}$ ]FAAGM was advanced to *in vivo* PET imaging in non-human primates but failed to show binding specificity in any of the analyzed brain regions despite excellent brain permeability, while autoradiography in human brain tissues revealed preferential binding to white matter, not blockable by either agomelatine or melatonin. We conclude that this is likely due to the poor pharmacokinetic properties of our lead molecules, including the fast metabolism *in vivo* and excessive non-specific binding due to their suboptimal lipophilicity. Low expression levels of both types of melatonin receptors in mammals in large structures of the brain and small anatomical sizes of the nuclei known to express the highest density of these receptors (e.g., SCN, ME, and pars tuberalis) may also contribute to the lack of specific binding of the tracer candidates. In addition, the binding of radioligands to melatonin receptors can be influenced by the fluctuation of their expression during the day–night cycle. While we acquired the data on the microsomal stability of the tracer candidates in the presence of liver microsomes, our study lacks the arterial input function, which prevented us from measuring the concentration of the parent tracer in blood during PET scans. This limits our ability to fully interpret the PET imaging data. Although PET imaging can play a valuable role in studies of the melatonergic system and drug development for the treatment of sleep, pain, and mood disorders, further efforts are required to develop selective melatonin PET tracers with more favorable pharmacokinetic properties, including lower lipophilicity and improved metabolic stability.

## Methods

**Chemistry. General.** Unless otherwise stated, all reagents were purchased from commercial sources (Sigma-Aldrich, VWR, and Fisher Scientific) and used without further purification. UCM765 has been kindly provided by Prof. Gilberto Spadoni's lab, University of Urbino, Italy. UCM1014 was synthesized as previously described starting from quinolin-5-ol in a 19% yield over five steps.<sup>43</sup> *N*-[2-(3-bromo-7-methoxy-naphthalen-1-yl)ethyl]acetamide was synthesized as previously described by bromination of agomelatine in acetic acid.<sup>44</sup> 3-Fluoroagomelatine has been kindly provided by Prof. SaïdYous's lab, University of Lille, France.

Flash column chromatography was carried out using silica gel 60 Å (particle size 40–63 µm, Silicycle, Canada) as the stationary phase. Compounds were visualized under UV light; compounds which could not be visualized with UV light were treated with the  $\text{KMnO}_4$  stain developer. All  $^1\text{H}$ ,  $^{13}\text{C}$ , and  $^{19}\text{F}$  NMR spectra were recorded on a two-channel Bruker 400 or 500 MHz AVIIIHD instrument with an Ascend magnet equipped with a BBFO+ SmartProbe at a constant temperature of 298 K. Correlation spectroscopy, distortionless enhancement by polarization transfer, heteronuclear single quantum coherence, and heteronuclear multiple bond correlation experiments were used to aid structural determination and spectral assignment.

Chemical shifts ( $\delta$ ) are reported in parts per million (ppm) and referenced to the residual solvent peak. Coupling constants ( $J$ ) are reported in hertz (Hz) to the nearest 0.5 Hz. Standard abbreviations indicating multiplicities are given: s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, m = multiplet, br = broad. High-resolution mass spectrometry (HRMS) data were obtained on a Bruker Maxis Impact Q-TOF by electrospray ionization–mass spectrometry (ESI-MS) in the positive or negative ionization mode. All chemicals used in this study were shown to be  $\geq 95\%$  pure by means of HPLC or NMR. *N*-(2-((3-Hydroxyphenyl) (phenyl)amino)ethyl)acetamide (*dm*UCM765). UCM765 (125 mg, 0.44 mmol) was dissolved in 3 mL of DCM and cooled to 0 °C.  $\text{BBr}_3$  (0.75 mL of 1 M solution in DCM, 1.7 equiv) was added slowly, and the reaction mixture was allowed to warm to room temperature and stirred for 18 h. The mixture was poured into water, neutralized with c.  $\text{NaHCO}_3$  solution, extracted with 3  $\times$  50 mL EtOAc, dried over  $\text{Na}_2\text{SO}_4$ , and then filtered, and the solvent was removed under reduced pressure. The crude product was purified by flash chromatography (silica, 2% MeOH/DCM) and isolated as 88 mg of pale pink glass in a 74% yield.  $^1\text{H}$  NMR (500 MHz,  $\text{DMSO}-d_6$ ):  $\delta$  9.22 (s, 1H), 7.96 (t,  $J$  = 5.7 Hz, 1H), 7.31–7.23 (m, 2H), 7.04 (m, 1H), 7.05–6.98 (m, 2H), 6.94 (m, 1H), 6.43 (m, 1H), 6.34 (m, 2H), 3.66 (dd,  $J$  = 8.0, 6.5 Hz, 2H), 3.21 (dt,  $J$  = 8.2, 6.1 Hz, 2H), 1.77 (s, 3H).  $^{13}\text{C}$  NMR (126 MHz,  $\text{DMSO}-d_6$ ):  $\delta$  169.5, 158.2, 148.7, 147.4, 129.9, 129.3, 121.3, 121.0, 110.8, 108.4, 107.2, 50.7, 36.3, 22.6. HRMS (ESI+)  $m/z$  calcd for  $\text{C}_{16}\text{H}_{18}\text{N}_2\text{NaO}_2$  [ $\text{M} + \text{Na}$ ] $^+$ ,

293.1260; found, 293.1257.

*N-((1-Benzyl-5-hydroxy-1,2,3,4-tetrahydroquinolin-2-yl)methyl)- propionamide (dmUCM1014)*. UCM1014 (50 mg, 0.15 mmol) was dissolved in 2 mL of DCM and cooled to 0 °C. 0.15 mL of BBr<sub>3</sub> (1 M in DCM, 1.7 equiv) was added slowly, and the reaction mixture was allowed to warm to room temperature and stirred for 18 h. The mixture was poured into water, neutralized with c. NaHCO<sub>3</sub> solution, extracted 3× with EtOAc, dried over Na<sub>2</sub>SO<sub>4</sub>, and then filtered, and the solvent was removed under reduced pressure. The crude product was purified by flash chromatography (silica, 0–10% MeOH/DCM) and isolated as 20 mg of pale brown glass in a 39% yield. <sup>1</sup>H NMR (500 MHz, DMSO-*d*<sub>6</sub>): δ 8.92 (s, 1H), 7.90 (t, *J* = 6.0 Hz, 1H), 7.29 (m, 2H), 7.25–7.14 (m, 3H), 6.63 (m, 1H), 6.06 (m, 1H), 5.86 (m, 1H), 4.65–4.41 (m, 2H), 3.46–3.38 (m, 1H), 3.22 (dt, *J* = 13.1, 5.2 Hz, 1H), 3.06 (ddd, *J* = 13.2, 9.2, 6.3 Hz, 1H), 2.73 (ddd, *J* = 17.0, 5.7, 2.2 Hz, 1H), 2.53–2.41 (m, 1H), 2.07 (q, *J* = 7.6 Hz, 2H), 1.95 (m, 1H), 1.70 (m, 1H), 0.99 (t, *J* = 7.6 Hz, 3H). <sup>13</sup>C NMR (126 MHz, DMSO-*d*<sub>6</sub>): δ 173.1, 154.7, 144.6, 139.5, 128.4, 126.5, 126.3, 126.2, 107.4, 102.8, 102.8, 56.3, 53.5, 39.5, 28.5, 21.7, 16.4, 9.9. HRMS (ESI+) *m/z* calcd for C<sub>20</sub>H<sub>23</sub>N<sub>2</sub>O<sub>2</sub> [M – H]<sup>–</sup>, 323.1765; found, 323.1768.

*2-Bromo-N-(2-(7-methoxynaphthalen-1-yl)ethyl)acetamide (BrAAGM)*. 2-(7-Methoxynaphthalen-1-yl)ethan-1-amine hydrochloride (59 mg, 0.25 mmol) was neutralized with saturated NaHCO<sub>3</sub> solution and then extracted 2× with CHCl<sub>3</sub>. The solution was dried over Na<sub>2</sub>SO<sub>4</sub> and filtered; then, the solvent was removed under reduced pressure to yield the free amine, which was used directly. The free amine was redissolved in 5 mL of DCM and cooled to 0 °C; then, 75 μL of bromoacetyl bromide (1.1 equiv) was added. The solution was warmed to room temperature and stirred for 30 min and then diluted with EtOAc and washed 1× with saturated NaHCO<sub>3</sub> and 1× with brine. The organic phase was dried over Na<sub>2</sub>SO<sub>4</sub> and filtered; then, the solvent was removed under reduced pressure. The crude product was purified by flash chromatography (silica, 0–40% EtOAc/ hexanes) to yield 66 mg of BrAAGM as a white solid in 98% yield. <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>): δ 7.77 (d, *J* = 8.9 Hz, 1H), 7.70 (dd, *J* = 7.6, 1.8 Hz, 1H), 7.45 (d, *J* = 2.4 Hz, 1H), 7.32–7.26 (m, 2H), 7.18 (dd, *J* = 8.9, 2.5 Hz, 1H), 6.62 (br s, 1H), 3.99 (s, 3H), 3.86 (s, 2H), 3.69–3.63 (m, 2H), 3.28 (t, *J* = 7.3 Hz, 2H). <sup>13</sup>C NMR (126 MHz, CDCl<sub>3</sub>): δ 165.7, 158.2, 133.2, 133.1, 130.5, 129.5, 127.4, 127.4, 123.3, 118.4, 102.4, 55.6, 40.7, 33.0, 29.3. HRMS (ESI+) *m/z* calcd for C<sub>15</sub>H<sub>16</sub>BrNNaO<sub>2</sub> [M + Na]<sup>+</sup>, 344.0257, 344.0250.

*2-Fluoro-N-(2-(7-methoxynaphthalen-1-yl)ethyl)acetamide (FAAGM)*. 2-(7-Methoxynaphthalen-1-yl)ethan-1-amine hydrochloride (115 mg, 0.48 mmol) was neutralized with saturated NaHCO<sub>3</sub> solution and then extracted 2× with CHCl<sub>3</sub>. The solution was dried over Na<sub>2</sub>SO<sub>4</sub> and filtered; then, the solvent was removed under reduced pressure to yield the free amine, which was used directly. The amine was dissolved in 3 mL of anhydrous toluene and added to a flask containing 19.1 mg of La(OTf)<sub>3</sub> (0.05 equiv) and 94.7 μL of ethyl fluoroacetate (1.2 equiv) according to the conditions in ref [49](#). The solution was heated at 55 °C for 48 h, cooled to room temperature, and then loaded directly onto a flash column (silica, 0– 40% EtOAc/hexanes). Pure FAAGM was isolated as 7 mg of a white solid in 19% yield. <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>): δ 7.77 (d, *J* = 8.9 Hz, 1H), 7.72–7.67 (m, 1H), 7.46 (d, *J* = 2.5 Hz, 1H), 7.34–7.27 (m, 2H), 7.18 (dd, *J* = 9.0, 2.5 Hz, 1H), 6.45 (s, 1H), 4.80 (d, *J* = 47.4 Hz, 2H), 3.99 (s, 3H), 3.72 (dt, *J* = 7.9, 6.4 Hz, 2H), 3.30 (t, *J* = 6.7 Hz, 2H). <sup>13</sup>C NMR (126 MHz, CDCl<sub>3</sub>): δ 168.0 (d, *J* = 17.0 Hz), 158.2, 133.2, 133.1, 130.5, 129.5, 127.4, 127.3, 123.3, 118.5, 102.4, 80.4 (d, *J* = 186.0 Hz), 55.6, 39.5, 33.4. <sup>19</sup>F NMR (471 MHz, CDCl<sub>3</sub>): δ –224.68 (td, *J* = 47.4, 3.3 Hz). HRMS (ESI+) *m/z* calcd for C<sub>15</sub>H<sub>16</sub>FNNaO<sub>2</sub> [M + Na]<sup>+</sup>, 284.1057; found, 284.1057.

*N-(2-(7-Methoxy-3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)naphthalen-1-yl)ethyl)acetamide (3-BpinAGM)*. Agomelatine (98 mg, 0.40 mmol) was dissolved in 2 mL of HOAc. To this, a solution of 25 μL of Br<sub>2</sub> (1.2 equiv) in 0.25 mL of HOAc was added, and the reaction mixture was heated at 70 °C for 6 h. The reaction mixture was cooled to room temperature, poured over ice, and stirred for 30 min; then, the resulting solution was extracted 2× with Et<sub>2</sub>O, washed once with saturated sodium thiosulfate solution, dried over Na<sub>2</sub>SO<sub>4</sub>, and filtered, and the solvent was removed under reduced pressure. The crude product was purified by flash chromatography (silica, 0– 2% MeOH/DCM) to furnish 3-BrAGM as 112 mg of an off-white powder in an 87% yield. <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>): δ 7.81 (m, 1H), 7.65 (m, 1H), 7.47 (m, 1H), 7.36 (m, 1H), 7.17 (m, 1H), 5.68 (br s, 1H), 3.98 (s, 3H), 3.57 (dt, *J* = 8.5, 6.2 Hz, 2H), 3.21 (t, *J* = 7.4 Hz, 2H), 1.97 (s, 3H). <sup>13</sup>C NMR (126 MHz, CDCl<sub>3</sub>): δ 170.6, 158.4, 136.2, 132.1, 130.5, 130.1, 129.4, 129.0, 119.7, 116.6, 102.8, 55.7, 40.3, 33.2, 23.5. HRMS (ESI+) *m/z* calcd for C<sub>15</sub>H<sub>16</sub>BrNNaO<sub>2</sub> [M + Na]<sup>+</sup>, 344.0257; found, 344.0254.

A vial was charged with 3-BrAGM (50 mg, 0.16 mmol), 49 mg of B<sub>2</sub>pin<sub>2</sub> (1.25 equiv), 23 mg of PdCl<sub>2</sub>dppf



(0.2 equiv), and 30 mg of KOAc (2 equiv). The vial was purged with argon; then, 3 mL of degassed DMSO was added, and the mixture was heated at 80 °C for 18 h. The reaction mixture was cooled to room temperature, then diluted into water, and washed 3× with EtOAc. The organic phase was washed 2× with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, and filtered, and the solvent was removed under reduced pressure. The crude product was purified by flash chromatography (silica, 0–80% EtOAc/hexanes) to yield 3-BpinAGM as 39 mg of a pale-brown solid in a 68% yield. <sup>1</sup>H NMR (500 MHz, DMSO-*d*<sub>6</sub>): δ 8.12 (t, *J* = 6.4 Hz, 1H), 8.11 (s, 1H), 7.92 (d, *J* = 9.0 Hz, 1H), 7.63 (d, *J* = 2.5 Hz, 1H), 7.52 (d, *J* = 1.2 Hz, 1H), 7.17 (dd, *J* = 8.9, 2.4 Hz, 1H), 3.96 (s, 3H), 3.32–3.27 (m, 1H), 3.15–3.09 (m, 2H), 1.83 (s, 3H), 1.32 (s, 12H). <sup>13</sup>C NMR (126 MHz, DMSO-*d*<sub>6</sub>): δ 169.5, 158.6, 134.8, 134.8, 133.4, 131.3, 131.0, 128.2, 118.2, 102.6, 83.6, 55.4, 39.8, 33.1, 24.8, 22.6. HRMS (ESI+) *m/z* calcd for C<sub>21</sub>H<sub>28</sub>BNNaO<sub>4</sub> [M + Na]<sup>+</sup>, 392.2004; found, 392.2001.

**Radiochemistry. General.** All radiosynthesis and quality control procedures were conducted in the cyclotron and radiochemistry facility of the McConnell Brain Imaging Centre (BIC), Montreal Neurological Institute (the Neuro), McGill University (Montreal, Canada), or in the cyclotron and radiochemistry facility of the University of Michigan (Ann Arbor, USA). Unless otherwise stated, reagents and solvents were commercially available and used without further purification: sodium chloride solution, 0.9% USP grade, and sterile water for the injection USP grade were purchased from Hospira or Baxter; ethanol was purchased from Commercial Alcohols (McGill University) or American Regent (University of Michigan); HPLC grade acetonitrile was purchased from Fisher Scientific. Other synthesis components were obtained as follows: sterile filters were obtained from Millipore; sterile product vials were purchased from Hollister-Stier; QMA-light and C18-light Sep-Paks were purchased from Waters Corporation. Radiosyntheses and HPLC purifications were carried out on the automated radiosynthesis units (Synthra for carbon-11 labelling at McGill University; Scintomics GRP for fluorine-18 labeling at McGill University; GE TRACERLAB FX<sub>FN</sub> for fluorine-18 labeling at the University of Michigan) equipped with semi-preparative HPLC columns. Collected tracer fractions were diluted with water (15 mL) and loaded on a preconditioned (5 mL of ethanol followed by 10 mL of water) Sep-Pak C-18 Light cartridge. The cartridge was washed with distilled water (5 mL), and the tracer was eluted with ethanol (0.5 mL), followed by saline (4.5 mL) into a vial. Quality control was performed on an analytical HPLC system (Agilent 1200 series at McGill University; Shimadzu Prominence UFLC at the University of Michigan) equipped with UV and radioactivity detectors using Phenomenex Aeris Peptide XB-C18 (250 × 4.6 mm, 3.6 μm), Phenomenex Prodigy ODS-3 (250 × 4.6 mm, 10 μm), or Phenomenex Luna PFP(2) (150 × 4.6 mm, 5 μm) columns. Radiotracer identities were confirmed by coinjection with the corresponding non-radioactive standards (Figures S1–S4).

**[<sup>11</sup>C]UCM765 and [<sup>11</sup>C]UCM1014.** [<sup>11</sup>C]Methyl iodide ([<sup>11</sup>C]CH<sub>3</sub>I) was produced *via* the gas-phase method and trapped in a solution of a dmUCM765 or dmUCM1014 precursor (1.0–2.0 mg) in DMSO (0.5 mL) containing 5 M NaOH (10 μL), followed by heating at 90 °C for 5 min. The reaction mixture was then diluted with 2 mL of the HPLC eluent and purified on a Phenomenex Luna C18 column (100 Å, 250 × 10 mm, 10 μm) using 20 mM NH<sub>4</sub>HCO<sub>2</sub>/MeCN (50:50) as an eluent at a 4 mL/min flow rate. Quality control HPLC (column: Phenomenex Aeris PEPTIDE 3.6 μm XB-C18, 250 × 4.6 mm; eluent: 0.1% TFA/MeCN (35:65); flow rate: 0.7 mL/min; wavelength: 300 nm) confirmed that [<sup>11</sup>C]-UCM765 and [<sup>11</sup>C]UCM1014 were produced with the RCPs of >99% and molar activities (*A<sub>m</sub>*) of 372 ± 60 GBq/μmol. Decay-corrected RCYs starting from [<sup>11</sup>C]CH<sub>3</sub>I were as follows: [<sup>11</sup>C]-UCM765 (17–22%, *n* = 3); [<sup>11</sup>C]UCM1014 (18%, *n* = 1). The syntheses were completed within 20–25 min from the completion of [<sup>11</sup>C]methyl iodide transfer.

**[<sup>18</sup>F]3FAGM. McGill Procedure.** [<sup>18</sup>F]F<sup>−</sup>/H<sub>2</sub>O (7.4–18.5 GBq) was passed through an unconditioned Sep-Pak Light 46 mg QMA cartridge (Waters) from the male side. The cartridge was then flushed with methanol (3 mL) from the male side, and <sup>18</sup>F-fluoride was then eluted with 450 μL of a tetraethylammonium bicarbonate solution in methanol (1 mg/mL), followed by methanol (500 μL) from the female side into a conical Wheaton vial (5 mL). The solvent was removed at 100 °C under vacuum first under a stream of argon (100 mL/min) and then in a closed system, and the vacuum was quenched with air. The reaction vial was then charged with the 3-BpinAGM precursor (3.7 mg, 10 μmol) and Cu(OTf)<sub>2</sub>py<sub>4</sub> (6.8 mg, 10 μmol) in a mixture of dimethylacetamide (500 μL) and 1-butanol (250 μL), and the mixture was allowed to react for 20 min at 130 °C. The crude mixture was then cooled to 50 °C, diluted with the HPLC eluent (1.5 mL), and purified on a Phenomenex Luna PFP(2) column (100 Å, 250 × 10 mm, 5 μm) using 20 mM NH<sub>4</sub>HCO<sub>2</sub>/MeCN (65:35) as an eluent at a 4 mL/min flow rate (*R<sub>t</sub>* = 33–35 min). The fraction containing the purified radiotracer was collected, reformulated as described above, and subjected to analytical HPLC procedures (column: Phenomenex Prodigy ODS-3, 250 × 4.6 mm; eluent: 0.1% TFA/MeCN (50:50); flow rate: 0.7 mL/min; wavelength: 230 nm). [<sup>18</sup>F]3FAGM was obtained within 90 min from [<sup>18</sup>F]F<sup>−</sup> in 20.3 ± 6.6% decay-corrected RCY (*n* = 2), >99% RCP, and 108.7 ± 47.5 GBq/μmol *A<sub>m</sub>*.

*University of Michigan Procedure.* The [ $^{18}\text{F}$ ]fluoride ion was produced using the  $^{18}\text{O}(\text{p},\text{n})^{18}\text{F}$  nuclear reaction *via* proton bombardment of a  $\text{H}_2^{18}\text{O}$  liquid target on a GE PETtrace cyclotron at a beam current of 55  $\mu\text{A}$  for 30 min. The [ $^{18}\text{F}$ ]fluoride from 2.5 mL of irradiated  $^{18}\text{O}$ -water was trapped on a QMA-light Sep-Pak cartridge (Waters) that had been preconditioned with ethanol (10 mL), 0.5 M KOTf aqueous solution (10 mL), and MilliQ  $\text{H}_2\text{O}$  (10 mL). Fluoride was eluted into the reaction vessel using a solution of tetraethylammonium bicarbonate (0.05 mg) and KOTf (10 mg) in 0.5 mL of water; the fluoride was then treated with acetonitrile (1 mL) and azeotropically dried at 100 °C first under vacuum and then under vacuum and argon overpressure. A solution of the 3-BpinAGM precursor (3.7 mg, 10  $\mu\text{mol}$ ) and  $\text{Cu}(\text{OTf})_2\text{py}_4$  (6.8 mg, 10  $\mu\text{mol}$ ) in a mixture of DMA (300  $\mu\text{L}$ ) and 1-butanol (150  $\mu\text{L}$ ) was added, and the reaction mixture was heated at 130 °C for 20 min. After cooling to 50 °C, the reaction mixture was diluted with the mobile phase (1.5 mL) and purified by semi-preparative HPLC (column: Luna PFP(2) 250  $\times$  10 mm; mobile phase: 20 mM  $\text{NH}_4\text{HCO}_2/\text{MeCN}$  (65:35) as an eluent at a 4 mL/min flow rate). The collected peak was diluted in 50 mL of MQ  $\text{H}_2\text{O}$  for reformulation. The solution was passed over a C18 1 cc Sep-Pak to trap the radiotracer and rinsed with water (5 mL) to remove the residual solvent. Elution with USP ethanol (1 mL) of the radiotracer into the collection vial was followed by rinsing of the Sep-Pak with saline (9 mL) to achieve a 10% ethanol in saline dose formulation that was passed through a sterile filter into a sterile vial prior to quality control analysis and use in imaging studies. The overall synthesis time was 105 min from the end of bombardment. Decay corrected product yield:  $7.95 \pm 0.77$  GBq ( $n = 2$ ), RCY = 11.5%,  $A_m = 159.89$  GBq/ $\mu\text{mol}$ .

*[ $^{18}\text{F}$ ]FAAGM. McGill Procedure.* [ $^{18}\text{F}$ ]F $^-/\text{H}_2\text{O}$  (7.4–18.5 GBq) was passed through a Sep-Pak Light Carbonate QMA cartridge (Waters), preconditioned with 10 mL of water. [ $^{18}\text{F}$ ]fluoride was then eluted with a mixture of aqueous  $\text{K}_2\text{CO}_3$  (0.125M, 150  $\mu\text{L}$ ), K222 (10–12 mg), and acetonitrile (~1 mL) into a conical Wheaton vial (5 mL). The solvent is azeotropically removed at 100 °C under vacuum and a stream of argon (100 mL/min), followed by two additional azeotropic drying steps with 0.5 mL of acetonitrile added each time. The reaction vial was then charged with the BrAAGM precursor (4.5 mg, 14  $\mu\text{mol}$ ) in acetonitrile (500  $\mu\text{L}$ ), and the mixture was heated for 15 min at 100 °C. The crude mixture was then cooled, diluted with the HPLC eluent (1.5 mL), and purified on a Phenomenex Luna C18 column (100 Å, 250  $\times$  10 mm, 10  $\mu\text{m}$ ) using 20 mM  $\text{NH}_4\text{HCO}_2/\text{MeCN}$  (60:40) at a 4 mL/min flow rate ( $R_t = 24$ –26 min). The fraction containing the purified radiotracer was collected, reformulated as described above, and subjected to analytical HPLC procedures (column: Phenomenex Prodigy ODS-3, 250  $\times$  4.6 mm; eluent:  $\text{MeCN}/\text{H}_2\text{O}$  (60:40); flow rate: 1.0 mL/min; wavelength: 230 nm). [ $^{18}\text{F}$ ]FAAGM was obtained within 90 min from [ $^{18}\text{F}$ ]F $^-$  in  $10.7 \pm 6.6\%$  decay-corrected RCY ( $n = 2$ ), >99% RCP, and  $281.9 \pm 147.5$  GBq/ $\mu\text{mol}$   $A_m$  ( $n = 2$ ).

*University of Michigan Procedure.* The [ $^{18}\text{F}$ ]fluoride ion was produced using the  $^{18}\text{O}(\text{p},\text{n})^{18}\text{F}$  nuclear reaction *via* proton bombardment of a  $\text{H}_2^{18}\text{O}$  liquid target on a GE PETtrace cyclotron at a beam current of 55  $\mu\text{A}$  for 30 min. The [ $^{18}\text{F}$ ]fluoride from 2.5 mL of irradiated  $^{18}\text{O}$ -water was trapped on a QMA-light Sep-Pak cartridge (Waters) that had been preconditioned with ethanol (10 mL), 0.5 M  $\text{NaHCO}_3$  aqueous solution (10 mL), and MilliQ  $\text{H}_2\text{O}$  (10 mL). Fluoride was eluted into the reaction vessel using an aqueous solution of  $\text{K}_2\text{CO}_3$  (3.5 mg in 0.5 mL of water), and then, crypt-222 (15 mg in 1.0 mL of acetonitrile) was added, so the fluoride could be azeotropically dried at 100 °C first under vacuum and then under vacuum and argon overpressure. A solution of the BrAAGM precursor (4.5 mg) in anhydrous acetonitrile (400  $\mu\text{L}$ ) was added to the dried fluoride in the reactor, and the solution was heated at 100 °C for 15 min. After cooling to 50 °C, the reaction mixture was diluted with the mobile phase (1.5 mL) and purified by semi-preparative HPLC (column, Luna PFP(2) 250  $\times$  10 mm; mobile phase, 20 mM  $\text{NH}_4\text{HCO}_2/\text{MeCN}$  (65:35) eluent at a 4 mL/min flow rate). The collected product was diluted in MQ  $\text{H}_2\text{O}$  (50 mL) transferred across a C18 1 cc Sep-Pak to trap the radiotracer and rinsed with water (5 mL) to remove the residual solvent. The product was eluted with ethanol (1 mL), and the Sep-Pak was rinsed with saline (9 mL) to give a 10% ethanol saline-formulated dose that was passed through a sterile filter into a sterile vial prior to quality control analysis and use in imaging studies. The overall synthesis time was 90 min after the end of bombardment. Decay corrected product yield:  $0.43 \pm 0.31$  GBq, RCY = 0.93%,  $n = 6$ ,  $A_m = 45.1$  GBq/ $\mu\text{mol}$ .

**PET Imaging in Rats.** All experimental procedures were approved by the McGill University Animal Care Committee (UACC, protocol #2017-7914) and were in compliance with the guidelines of the Canadian Council on Animal Care (CCAC). Male *Sprague–Dawley* rats (300–400 g, 6–9 weeks old) were purchased from Charles River Canada for microPET imaging experiments. All PET images were acquired using a CTI Concorde R4 microPET scanner for small animals (CTI, Siemens, Munich, Germany) between 11 a.m. and 3 p.m. (Zeitgeber time = 6–10 h). Anesthesia was first induced using isoflurane at 5% with a 2–3 L/min oxygen flow and then maintained for the duration of the scan at 1.5–2.5% isoflurane with a 0.8 L/min oxygen flow. The rat brain was positioned at the center of field of view, and the 60-min dynamic scan was acquired in 27 frames (8  $\times$  30 s, 6  $\times$  1 min, 5  $\times$  2 min, 8  $\times$  300 s) concomitantly with the bolus injection of the tracer in 10% ethanol saline in the tail vein. The mean injected tracer activity was 17.5 MBq, and the mean injected volume was 0.7 mL. Each emission scan was either preceded (for  $^{18}\text{F}$ -labeled tracers) or followed (for  $^{11}\text{C}$ -labeled tracers) by a 9-min transmission scan with a  $^{57}\text{Co}$  point source to correct for attenuation. In blocking experiments, a solution of the competitor was injected either subcutaneously 7–10 min prior to the tracer injection (4P-PDOT, melatonin or agomelatine) or intravenously immediately before



tracer injection (non-radioactive UCM765). Magnetic resonance imaging (MRI) structural images for coregistration purposes were obtained using a 7 T Bruker Pharmascan preclinical MRI system, with a Bruker volume resonator radiofrequency coil designed for rat brain imaging. Images were reconstructed using the *Maximum A Posteriori* (MAP) algorithm and corrected for attenuation, scatter, dead time, and decay. Imaging processing and analyses were conducted using MINC tools ([www.bic.mni.mcgill.ca/ServicesSoftware](http://www.bic.mni.mcgill.ca/ServicesSoftware)). Both the eyes, the nose bone, and the base of the skull were used as landmarks to coregister the PET to MRI images. Atlas-based autosegmentation of the regions of interest (ROIs) was performed with ITK-SNAP (v. 3.6.0; <http://www.itksnap.org/pmwiki/pmwiki.php>)<sup>50</sup> based on brain region delineations reported in the Waxholm Space atlas of the Sprague–Dawley rat brain (<https://www.nitrc.org/projects/whs-sd-atlas>).<sup>51</sup> Radioactivity counts were normalized to the amount of injected activity and animal weight, and the results were expressed as SUVs

$$\text{SUV} = \frac{\text{radioactivity counts} \times \text{body weight(g)}}{\text{injected dose(Bq)}}$$

**PET Imaging in Non-human Primates.** All primate imaging studies were performed in accordance with the standards set by the University Committee on Use and Care of Animals (UCUCA) at the University of Michigan. Imaging was done in two intact mature female rhesus monkeys (*Macaca mulatta*, body weight = 9.06–9.72 kg with negligible variation throughout the duration of the study) using the Concorde Microsystems MicroPET P4 tomograph (Knoxville, TN) between 10 a.m. and 12 p.m. (Zeitgeber time = 4–6 h). The animals were anesthetized in the home cage with Telazol and transported to the PET facility. Subjects were intubated for mechanical ventilation, and anesthesia was continued with isoflurane. Anesthesia was maintained throughout the duration of the PET scan. A venous catheter was inserted into one hind limb, and the monkey was placed on the PET gantry with its head secured to prevent motion artifacts. 10 minutes later, 0.16–0.17 GBq of [<sup>18</sup>F]FAAGM or [<sup>18</sup>F]3FAGM was administered in a bolus dose over 1 min, and the brain scan acquisition started concomitantly for 120 min. Emission data were collected in 19 frames (5 × 1 min, 2 × 2.5 min, 2 × 5 min, 10 × 10 min). Collection of vitals (HR, SPO<sub>2</sub>, EtCO<sub>2</sub>, and respiratory rate) was carried out during the whole scan. For the blocking scans, agomelatine (3.5 mg/mL formulation in 6.6% ethanol and 1.6% Tween-80 in saline) was passed through a sterile filter and injected intravenously at a 4 mg/kg dose immediately (no more than 10 min) before injection of the radiotracer. Emission data were corrected for attenuation and scatter and reconstructed using the 3D maximum a priori method (3D MAP algorithm). PET scans were registered to an existing FDG monkey PET scan from the University of Michigan PET facility using SPM (<https://www.fil.ion.ucl.ac.uk/spm/doc/>). The regions defined previously on a variety of PET radiotracer scans, including FDG, flumazenil, and carfentanil, were applied to the scans reported in this paper. Using a summed image of the entire data set, ROIs were drawn by alignment with the NIMH macaque brain atlas ([https://afni.nimh.nih.gov/pub/dist/doc/htmldoc/nonhuman/\\_macaque\\_templat/template\\_nmtv2.html](https://afni.nimh.nih.gov/pub/dist/doc/htmldoc/nonhuman/_macaque_templat/template_nmtv2.html)) per published methods.<sup>52</sup> Data from multiple planes were obtained for volumetric ROIs for the whole brain, thalamus, hypothalamus, pituitary gland, cerebellum gray and white matter, and BG. The volumetric ROIs were then applied to the full dynamic data sets to obtain the regional tissue time–radioactivity data.

**Autoradiography.** Human brain tissues were acquired from the Douglas-Bell Canada Brain Bank and used under approved protocol #IUSMD-20-02 to Rosa-Neto. Rat brain tissues were acquired from the sacrificed male Long–Evans rats (400–500 g, 11 months old, Charles River Canada) by decapitation and extraction, followed by the rapid freezing of the brains under approved protocol #7301 to Rosa-Neto. The tissues frozen to –80 °C were sliced to a 20 µm thickness and mounted onto Superfrost Plus slides (Thermo Scientific). The aqueous buffer was prepared from a solution of NaCl (138 mM) and KCl (2.7 mM) by adjusting the pH to 7.4 with NaOH and adding bovine serum albumin (1%). After thawing and preincubation in either the buffer (17 mL) or a blocking solution (10 µM, 17 mL) for 1 h, the solution of the radiotracer was added to bring the radioactivity concentration to 37 kBq/mL and the slides were incubated in the tracer solution with or without the competitor for an additional 40 min (for <sup>11</sup>C-labeled tracers) or 2.5 h (for <sup>18</sup>F-labeled tracers). After a rinse with the cold buffer (3 × 3 min, 0 °C) followed by cold water (30 s, 0 °C), the labeled slices were dried and placed on phosphor-imaging plates with industrial tritium activity standards.

After exposure for 20 min, the plates were scanned with a plate reader (BAS 5000; Fuji or Typhoon Trio + Variable Mode Imager).

**In Vitro Biological Assays.** Plasma protein binding, stability in the presence of rat liver microsomes, and log *D*<sub>7.4</sub> and bidirectional permeability assessment in the MDCK–mdr1 transfected line of 3FAGM and FAAGM were performed at the contract research organization (CRO) Paraza Inc. following standard protocols.

**Statistical Analyses.** A two-way paired sampled *t*-test was conducted using SPSS software to determine the difference between saline- and blocking agent (agomelatine or MLT)-induced changes in the area under curve of tracers' uptake.

## Acknowledgements

The authors thank Robert Hopewell, I-Huang Tsai, Marina Kostikova, and Monica Samoila for access to medical radioisotopes and assistance with tracer radiosynthesis and quality control. The authors thank Paraza Inc. for performing *in vitro* biological assays on tracer candidates.

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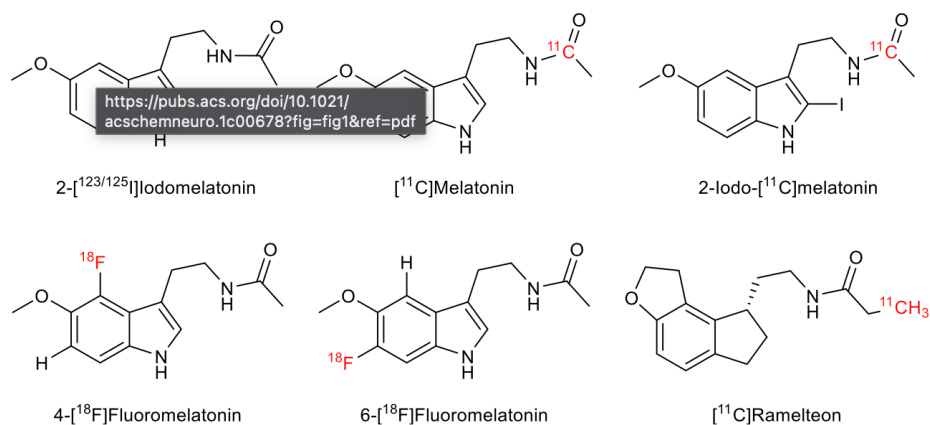
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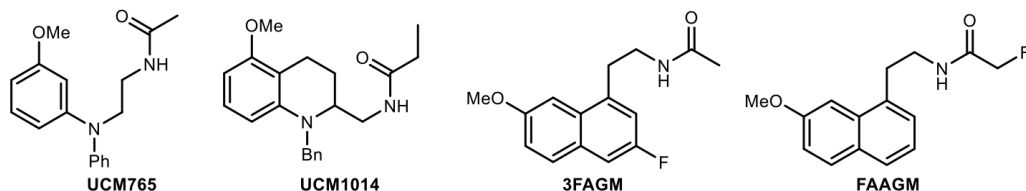
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## FIGURES AND TABLES



**Figure 1.** Previously developed melatonergic radiotracers.

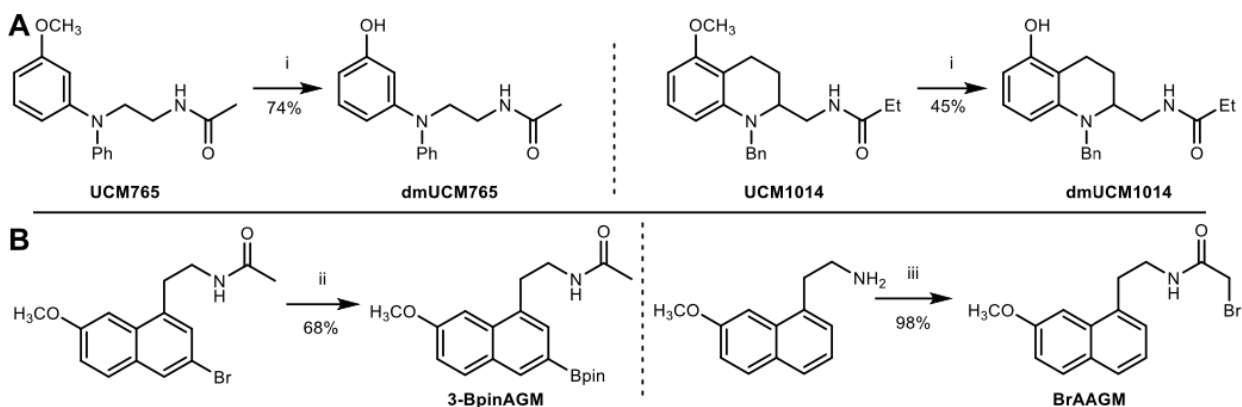


**Figure 2.** Structures of the reported MT ligands selected for radiolabeling with carbon-11 or fluorine-18.

**Table 1.** Affinities to MT<sub>1</sub>/MT<sub>2</sub> and CNS MPO Scores of the Selected Ligands

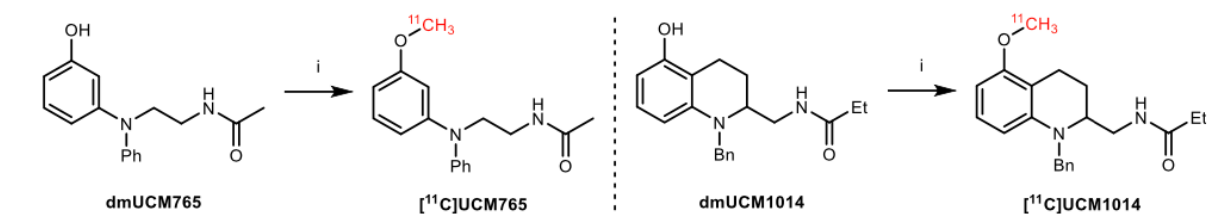
ligand	K <sub>i</sub> MT <sub>1</sub> , nM	K <sub>i</sub> MT <sub>2</sub> , nM	CNS MPO
UCM765 <sup>39</sup>	4.2	0.066	5.58
UCM1014 <sup>43</sup>	17	0.001	4.42
3FAGM <sup>44</sup>	0.1	0.07	5.66
FAAGM <sup>44</sup>	0.07	0.07	5.69

**Scheme 1. (A) Syntheses of Desmethyl Precursors for  $^{11}\text{C}$ -Labeled Tracers Based on UCM Ligands; (B) Syntheses of Precursors for  $^{18}\text{F}$ -Labeled Tracers Based on Agomelatine Derivatives<sup>a</sup>**



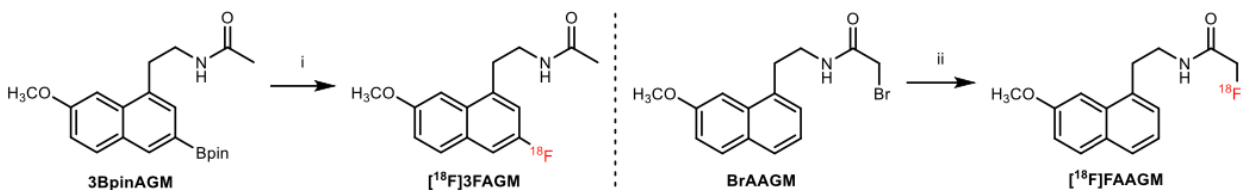
<sup>a</sup>(i)  $\text{BBr}_3$ , DCM, 0 °C–r.t., 18 h; (ii)  $\text{B}_2\text{pin}_2$ ,  $\text{PdCl}_2(\text{dppf})$ , KOAc, DMSO, 80 °C, 18 h; (iii)  $\text{BrCH}_2\text{COBr}$ ,  $\text{CHCl}_3$ , r.t., 30 min.

**Scheme 2. Radiosynthesis of  $[^{11}\text{C}]\text{UCM765}$  and  $[^{11}\text{C}]\text{UCM1014}$  as PET Tracer Candidates for Selective Imaging of  $\text{MT}_2$ <sup>a</sup>**

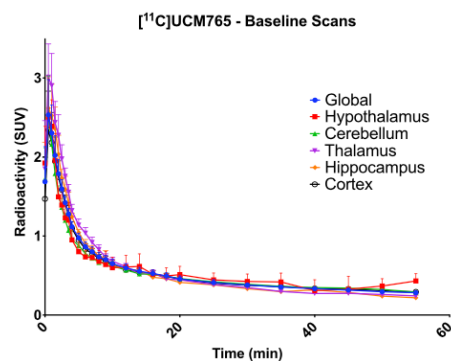


<sup>a</sup>(i)  $[^{11}\text{C}]\text{CH}_3\text{I}$ , DMSO, 5 M NaOH, 90 °C, 5 min.

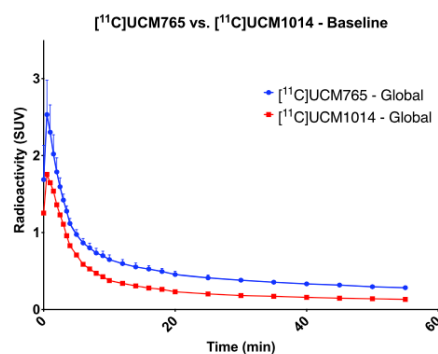
**Scheme 3. Radiosynthesis of  $[^{18}\text{F}]\text{3FAGM}$  and  $[^{18}\text{F}]\text{FAAGM}$  as PET Tracer Candidates for Imaging of  $\text{MT}_1$  and  $\text{MT}_2$ <sup>a</sup>**



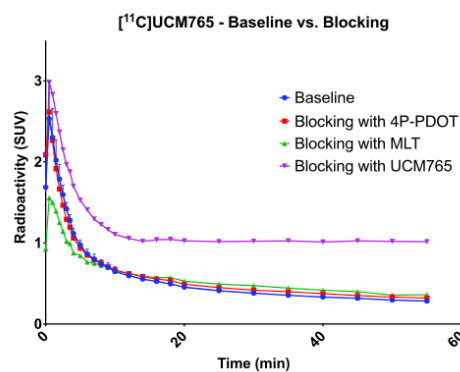
<sup>a</sup>(i)  $[^{18}\text{F}]\text{Et}_4\text{NF}$ ,  $\text{Cu}(\text{OTf})_2\text{py}_4$ , DMA/1-BuOH, 130 °C, 20 min; (ii)  $[^{18}\text{F}]\text{KF}$ ,  $\text{K}_2\text{CO}_3/\text{K}_{222}$ , MeCN, 100 °C, 15 min.



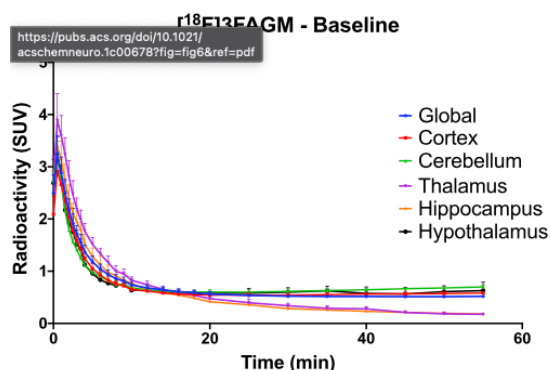
**Figure 3.** Time-activity curve of the brain regional uptake of  $[^{11}\text{C}]\text{UCM765}$  in wild-type Sprague-Dawley rats ( $n = 4$ ).



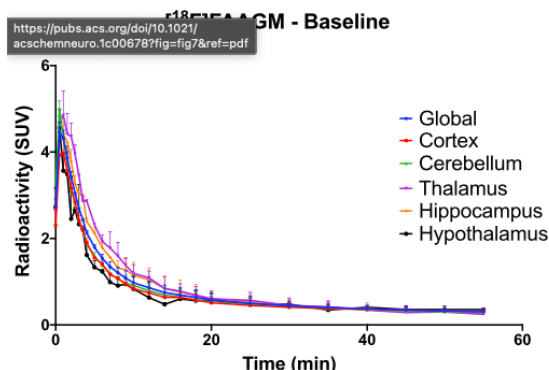
**Figure 4.** Time-activity curves of global cerebral uptake of  $[^{11}\text{C}]\text{UCM765}$  (blue;  $n = 4$ ) and  $[^{11}\text{C}]\text{UCM1014}$  (red;  $n = 1$ ) in wild-type Sprague-Dawley rats.



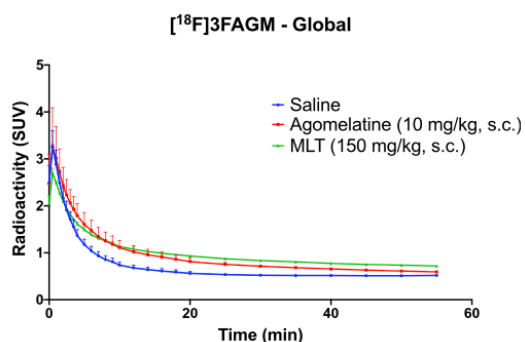
**Figure 5.** Time-activity curve of the brain regional uptake of  $[^{11}\text{C}]\text{UCM765}$  cerebral uptake following an injection of saline (blue, s.c.,  $n = 4$ ), 4P-PDOT (red, 10 mg/kg, s.c.,  $n = 1$ ), MLT (green, 150 mg/kg, s.c.,  $n = 1$ ), or non-radioactive UCM765 (purple, 20 mg/kg, i.v.,  $n = 1$ ) in wild-type Sprague-Dawley rats.



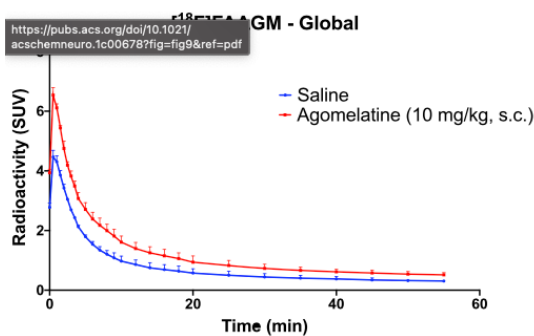
**Figure 6.** Time-activity curve of the brain regional uptake of  $[^{18}\text{F}]\text{3FAGM}$  in wild-type Sprague-Dawley rats ( $n = 4$ ).



**Figure 7.** Regional baseline cerebral uptake of  $[^{18}\text{F}]\text{FAAGM}$  in wild-type Sprague-Dawley rats ( $n = 2$ ).

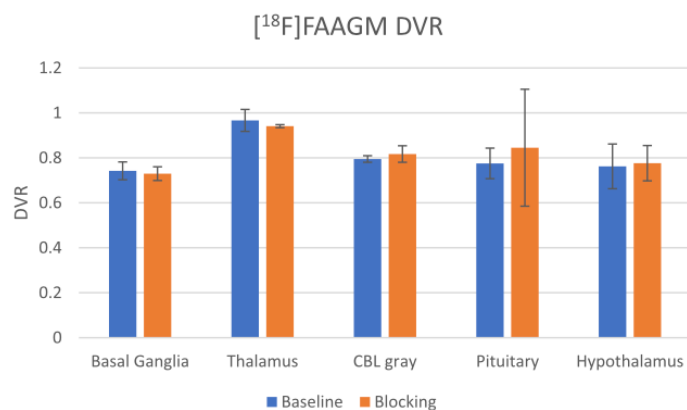


**Figure 8.** Blocking of  $[^{18}\text{F}]\text{3FAGM}$  with either agomelatine ( $n = 2$ ) or melatonin ( $n = 2$ ) does not result in reduced tracer uptake in wild-type Sprague-Dawley rats.



**Figure 9.** Blocking of  $[^{18}\text{F}]\text{FAAGM}$  with agomelatine ( $n = 2$ ) does not result in reduced tracer uptake in wild-type Sprague-Dawley rats.





**Figure 10.** DVRs of [ $^{18}\text{F}$ ]FAAGM in five different brain regions with cerebellum white matter as a reference region show preferential accumulation of the tracer in the white matter and a lack of specific binding in blocking experiments ( $n = 2$ ) with agomelatine (4 mg/kg) in rhesus monkeys.

**Table 2.** Liver Microsomal Stability (Rat), Microsomal Clearance, Plasma Protein Binding (Rat),  $\log D_{7.4}$ , and Bidirectional Permeability through MDCK-mdr1 and the Efflux Ratio of FAAGM and 3FAGM

compound	$t_{1/2}$ (min) in liver microsomes (rat)	$Q_h$ , %	plasma protein binding, %	$\log D_{7.4}$	efflux ratio BA/AB
FAAGM	$9.3 \pm 0.4$	79.3	$90.0 \pm 0.5$ , %	$2.68 \pm 0.05$	1.3
3FAGM	$7.5 \pm 0.9$	82.6	$92.8 \pm 0.7$ , %	$2.97 \pm 0.01$	1.2