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# Enabling efficient PET imaging of Synaptic Vesicle glycoprotein 2A (SV2A) with a robust and one-step radiosynthesis of a highly potent <sup>18</sup>F-labelled ligand ([<sup>18</sup>F]UCB-H)

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Abstract: We herein describe the straightforward synthesis of a stable pyridyl(4methoxyphenyl)iodonium salt and its [ $^{18}$ F]radiolabelling within a one-step, fully automated and cGMP compliant radiosynthesis of [ $^{18}$ F]UCB-H ([ $^{18}$ F]7), a PET tracer for the imaging of Synaptic Vesicle glycoprotein 2A (SV2A). Over the course of one year, 50 automated productions provided  $34\pm2\%$  of injectable [<sup>18</sup>F]7 from up to 285 GBq (7.7 Ci) of [<sup>18</sup>F]fluoride in 50 minutes (uncorrected radiochemical yield. Specific Activity =  $815\pm185$  GBq/µmol). The successful implementation of our synthetic strategy within routine, high-activity and cGMP productions attests to its practicality and reliability for the production of large doses of [<sup>18</sup>F]7. In addition to enabling efficient and cost-effective clinical research on a range of neurological pathologies through the imaging of SV2A, this work further demonstrates the real value of iodonium salts for the cGMP <sup>18</sup>F-PET tracer manufacturing industry, and their ability to fulfill practical and regulatory requirements in that field.

#### INTRODUCTION

[<sup>18</sup>F]UCB-H<sup>1,2</sup> ([<sup>18</sup>F]7, Figure 1) is a novel Positron Emission Tomography (PET) tracer that has nanomolar affinity for the Synaptic Vesicle glycoprotein 2A. The pivotal role of SV2A in neurotransmission processes and its proven implication in epilepsy make it a highly interesting PET target for the study of various neurotransmission-related diseases, such as epilepsy and Alzheimer's disease.<sup>3–5</sup>

Figure 1. Two SV2A ligands: the anti-epileptic Levetiracetam and the PET tracer  $[^{18}F]$ 7.



Epilepsy alone is a neurological pathology that affects over 50 million people around the globe at any given time,<sup>6</sup> and yet the cellular processes it originates from still remain vastly unknown and unexplored. The ubiquity of SV2A in the human brain, however, offers a unique opportunity

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to peek into the very mechanisms of neurotransmission and improve the understanding, diagnosis and treatment of epilepsy and other neurological conditions using PET technology. PET has indeed become a corner stone tool for (pre-)clinical research and routine diagnosis over the past decades;<sup>7–10</sup> with its convenient 110 min half-life, almost exclusive  $\beta$ + decay and the significant representation of fluorine in pharmacologically active molecules,<sup>11</sup> fluorine-18, in its no-carrier-added, nucleophilic ([<sup>18</sup>F]F<sup>-</sup>) form, is one of the most practical nuclides currently in use for clinical PET imaging.<sup>12</sup>

To this day, however, designing an efficient and robust automated cGMP production method for an aromatic  $[^{18}F]$ -labelled compound such as  $[^{18}F]$ 7 remains a challenge, due to the unique reactivity of fluoride ion. A strong electron withdrawing group in ortho or para of an appropriate leaving group (typically  $-N(Me)_3^+$  or  $-NO_2$ ) is often a *sine qua non* condition for the efficient nucleophilic fluorination of an aromatic ring, which severely limits the substrate scope of the reaction. This approach was used in the first radiosynthesis of [18F]7 (Scheme 1), for its assessment as an efficient PET imaging agent of SV2A.<sup>4</sup> The relative complexity of the chemistry involved makes the multi-step synthesis very challenging to automate, and restricts its radiochemical yield and its robustness. Because of this poor synthetic accessibility, the syntheses of the <sup>11</sup>C-derived analogs of [<sup>18</sup>F]7, [<sup>11</sup>C]UCB-A and [<sup>11</sup>C]UCB-J, were recently developed (see Table 2).<sup>13,14</sup> The very short half-life of carbon-11 (~20 min) however implies less flexibility in terms of synthesis and medium-range delivery to PET centers and hospitals, and [<sup>18</sup>F]-labelled analogs are much preferred when possible. Considering the potential of  $[^{18}F]7$  in neurological research and the increasing demand for it in clinical trials, the development of a robust and effective synthesis was undertaken.



Scheme 1. The multi-step radiosynthesis first used for the production of  $[^{18}F]$ 7, and the novel strategy presented in this work.



Overcoming the intrinsic limitations of nucleophilic aromatic substitution has been the driving force of multiple research projects for the past few years. Several publications reported the development of a variety of novel precursors that allow the late-stage radiolabelling of electronrich aromatic rings, thereby potentially enabling considerable upgrades in the synthesis of [<sup>18</sup>F]fluoroarene-derived radiotracers. Iodonium salts,<sup>15</sup> iodonium ylides,<sup>16–18</sup> boronic esters,<sup>19</sup> palladium<sup>20</sup> or nickel<sup>21</sup> complexes have been proposed as versatile precursors for direct nucleophilic aromatic radiofluorination. However, some of those precursors have, at this stage of their development, physico-chemical properties that make them less suited than others for automated and cGMP productions of PET tracers. Iodonium salts, for their part, have repeatedly been shown to be efficient and stable late-stage radiofluorination precursors from their first reported <sup>18</sup>F-radiolabelling in 1995, which prompted us to develop such an iodonium-based precursor for the one-step radiosynthesis of [<sup>18</sup>F]7.<sup>22-38</sup> To this day, little information has been made available on the routine, high-activity  $[^{18}F]$ -labelling of iodonium salts in the framework of cGMP productions; filling that gap was also one of the objectives of this project. We also tackled what may have represented a major drawback of iodonium  $[^{18}F]$ -radiolabellings in a cGMP

perspective, which is the use of the genotoxic radical scavenger TEMPO ((2,2,6,6-Tetramethylpiperidin-1-yl)oxy) for controlling the radical side-reactions occurring during the radiolabelling, and increasing the yield and reproducibility thereof.<sup>31,39,40</sup>

#### **RESULTS & DISCUSSION**

#### Precursor synthesis & characterization

Although a number of reactions allow the synthesis of diaryliodonium salts,<sup>30,41–46</sup> few are compatible with the synthesis of pyridyl(aryl)iodoniums. Recent developments in that field now allow their one-step synthesis from the corresponding iodinated heterocyle.<sup>41,42</sup> The method only requires common and fairly inexpensive reagents, and no transition metal compound is needed. In a cGMP perspective, the absence of metallic impurities simplifies the mandatory quality control of the obtained precursor. Our synthetic strategy for pyridyl(4-methoxyphenyl)iodonium salt **6** is depicted in Scheme 2.

Scheme 2. Synthesis of 6 from compounds 1 and 4a. The overall yield exceeds 30%.



We started from commercial methyl-3-aminoisonicotinate 1, to form 2 through a modified Sandmeyer reaction followed by reduction with DIBAL at -96 °C to yield aldehyde  $3.^{47,48}$ Special attention was dedicated to the temperature control of the  $2 \rightarrow 3$  reduction step (CH<sub>2</sub>Cl<sub>2</sub>/N<sub>2</sub> cooling bath), as complete reduction to the alcohol analog of 3 and hydrodehalogenation by-products became predominant at higher temperatures. Maximal reproducibility was observed when using methanol to neutralize the excess of DIBAL, which allowed the quenching to occur at -96 °C in a homogeneous medium. The following three-step, one-pot reaction converts **3** into **5** through a reductive amination with **4b** followed by an intramolecular cyclisation in basic conditions. The last step  $(5 \rightarrow 6)$  requires strongly oxidizing conditions (4 eq. of triflic acid on **5** followed by the addition of 2.5 eq. of *m*-CPBA), but proceeds with high and reproducible yields (75-80%). The overall synthesis proceeds with a very good yield (>30% overall) and does not involve any semi-prep HPLC purification, which makes it as practical as it is effective. It is noteworthy that all the reaction steps were optimized at least at a 10 mmol scale, except the last one  $(5 \rightarrow 6)$  that yields enough precursor (1.3 g) for more than 80 radiolabellings at a 2.5 mmol scale. The novel compound **6** was fully characterized (melting point, CHNS analysis, HRMS, NMR and X-Ray crystallography - Figure 2), and a validated cGMP <sup>1</sup>H-NMR quantification method was also developed for the assessment of its absolute purity (measured purity: 99.8±0.5 wt.%).

**Figure 2.** ORTEP representation of **6** with thermal ellipsoids drawn at the 50% probability level. Hydrogen atoms and counter-ion molecules were removed for clarity.



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In order to avoid a potentially costly enantiomeric resolution of the final product, the retention of the initial enantiomeric excess (ee) of **4a** during the whole synthetic process of **6** was of prime importance. By synthesizing **5** and **6** with both enantiomerically pure **4a** and racemic (*R*,*S*)-**4a**, we demonstrated that the critical basic cyclisation step ( $\mathbf{3} \rightarrow \mathbf{5}$ , scheme 2) did not cause any detectable racemization in compound **5**. We also showed that neither the last step ( $\mathbf{5} \rightarrow \mathbf{6}$ ), nor the radiolabelling conditions cause any measurable racemization of the stereogenic center (comparison of UV and radio chromatograms by chiral HPLC. See SI and Figure 3).

The pyridyl(4-methoxyphenyl)iodonium salt **6** additionally displayed an exemplary stability in standard atmospheric conditions, which is a highly valuable feature in a routine and cGMP perspective. The projections of the ongoing stability study tend to confirm a precursor shelf-life of 2 years (storage conditions: crimp-capped amber vial kept at 5 - 8  $^{\circ}$ C).

#### Radiochemistry

In order to probe our precursor's inclination to undergo the expected radiofluorination, lowactivity labelling tests were carried out, using a Waters® 46 mg QMA carbonate for [<sup>18</sup>F]fluoride trapping and a  $K_{222}/K_2CO_3$ -based QMA eluent for the subsequent [<sup>18</sup>F]fluoride elution and azeotropic drying in the reactor. A solution of **6** and TEMPO in a polar aprotic solvent was added to the dry fluoride residue, and the reactor was then sealed and heated up to the given temperature and during the time given in Table 1. It appeared that usual concentrations of cryptand-2.2.2 (K<sub>222</sub>) and potassium carbonate in the QMA eluent were ineffective for the <sup>18</sup>Flabelling of the iodonium precursor **6** (Table 1, Entry 1). The precursor indeed displayed poor chemical stability in the presence of high concentrations of base (6.6 mg/mL K<sub>2</sub>CO<sub>3</sub>), which in turn led to poor radiochemical yields. We therefore tested the influence of the K<sub>2</sub>CO<sub>3</sub> and K<sub>222</sub> concentrations in the QMA eluent on the radiochemical yield and on the stability of the precursor.

 Table 1. Optimization of the labelling conditions of the pyridyl(4-methoxyphenyl)iodonium

 precursor 6.



[a] Amount contained in the QMA eluent (600  $\mu$ L of a 90/10 MeOH/H2O solution). [b] Using 15.5mg of **6**. [c] Decaycorrected radiochemical yield measured by radio-TLC, corrected for [<sup>18</sup>F]fluoride adsorption on the reactor walls.

Using qualitative UV-TLC monitoring and quantitative radio-TLC monitoring, we found that low concentrations of  $K_2CO_3$  enabled a better precursor lifetime in the reaction medium, thus allowing for more efficient radiolabellings (Entries 2-3). Diaryliodonium salts are known to decompose into radical intermediates upon heating,<sup>40</sup> leading to undesired by-products and to low, fluctuating radiochemical yields. The radical scavenger TEMPO was previously reported to be a very efficient agent for the enhancement of diaryliodonium [<sup>18</sup>F]radiolabellings.<sup>39</sup> In our case, it proved necessary for the efficient radiofluorination of **6**; 1 to 1.2 equivalent of TEMPO (with respect to **6**) provided the most efficient RCY (Entries 3-7), using MeCN as the solvent of the reaction (Entry 7). The optimization of the [<sup>18</sup>F]labelling parameters led to the following

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conditions, which were subsequently used for the automated productions:  $15.5\pm0.5$  mg of precursor and  $4.3\pm0.2$  mg of TEMPO were solubilized in 1 mL of dry acetonitrile and heated for 10 minutes at 125 °C in the presence of the azeotropically dried <sup>18</sup>F-fluoride, affording [<sup>18</sup>F]7 at 50% RCY. The UPLC retention time of the obtained labeled compound was compliant with that of the cold reference [<sup>19</sup>F]7. LC-MS detection of the cold peak associated with the radioactive peak (Observed m/z = 325 = M+1) further confirmed the identity of the radiotracer. The enantiomeric excess of the stereogenic center was left unchanged by the radiolabelling conditions, as shown in Figure 3. Additionally, although non-symmetric iodonium salts are known to undergo unselective radiofluorination in many cases,<sup>38</sup> we found that in our conditions, the radiolabelling was strictly regioselective for the pyridyl moiety of the pyridyl(4-methoxyphenyl)iodonium salt **6** (no [<sup>18</sup>F]4-Fluoroanisole was detected in the crude reaction mixture, see Supporting Information).

The purity of the iodonium precursor was found to be determinant of the radiochemical yield. Especially, the deprotonation step of the iodonium **6** on a basic alumina plug (which is a part of the work-up of its synthesis, see Experimental Section), should be carried out by strictly following the reported procedure.<sup>42</sup> While the incomplete deprotonation of the obtained iodonium cannot usually be detected on classical NMR spectra, it nevertheless strongly undermines the [<sup>18</sup>F]labelling yield, due to the presence of labile, nucleophilicity-poisoning protons. For instance, a precursor batch incorrectly submitted to the deprotonation step afforded significantly lower radiochemical yields (33% instead of 50%), although its <sup>1</sup>H- and <sup>13</sup>C-NMR spectra were strictly identical to other functional precursor batches. A second, more careful deprotonation procedure was performed on the defective batch, and the RCY was restored to its nominal value. Besides, the crystallization method for the precursor **6** presented in this work has

been extensively optimized and should also be followed closely, as it ensures obtaining a pure and highly soluble crystalline form of **6**, which in turn facilitates its solubilization during the automated radiosynthesis.

**Figure 3.** Chiral HPLC analysis of  $[^{19}F]7$  (blue trace), and of  $[^{18}F]7$  obtained from the radiolabelling of **6** and (*R*,*S*)-**6** (green and red traces respectively). Only the expected *R* enantiomer of  $[^{18}F]7$  is produced during the high-activity radiolabelling of **6** (no measurable racemization, green radio trace, n = 3).



#### Automation & productions at high activity

The radiosynthesis was automated on a Trasis All-In-One synthesizer equipped with an integrated HPLC purification system (Figure 4). The automated sequence is constituted of the following main steps: 1) Machine and cassette tests, HPLC and C18 formulation cartridge conditioning, preparation of the final formulation and precursor solubilization (pre-synthesis, 30

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min). 2) <sup>18</sup>F-Fluoride recovery, trapping, elution and drying (7 min). 3) Precursor transfer to the reactor, heating and radiolabelling (13 min). 4) Cooling of the reactor, dilution of the crude and injection on the semi-preparative HPLC (3 min). 5) HPLC purification and collection of the purified radiotracer (20 min). 6) Formulation of the radiotracer into an injectable solution (8 min). In the Experimental Section, we describe more precisely the string of events constituting the radiosynthesis. Extensive description of the cassette and of the materials used throughout the synthesis may be consulted in the Supporting Information and in the Experimental section.

**Figure 4.** Graphical representation of the cassette designed for the automated synthesis of [<sup>18</sup>F]**7** on a Trasis AIO synthesizer.



The automation allowed us to perform syntheses at high levels of activity and assert that the RCY remained constant at every tested starting activity (tested up to 7.7 Ci, or 285 GBq). A total of 50 fully automated productions, 40 of which were performed at high activities (typical starting activity = 110 GBq), yielded a narrow and reproducible  $34\pm2\%$  uncorrected RCY in [<sup>18</sup>F]7 with high specific activity ( $815\pm185$  GBq/µmol, n = 8). The RCY of the automated synthesis was defined as the ratio of the activity of [<sup>18</sup>F]7 present in the injectable solution at EOS (End Of the Synthesis) to the activity trapped on the QMA at SOS (Start Of Synthesis, *i.e.* 50 minutes before). The obtained regularity in the automated synthesis RCY's during a one-year production campaign with 4 different batches of precursor illustrates the robustness and reliability of this production method. In addition, the single reaction step renders the automation development quite straightforward in its general principle; this synthetic strategy may therefore be adapted on virtually any commercial synthesizer.

#### The Radiolysis Issue

While the amount of activity present in the reaction medium had no effect on the labelling yield, it had an important consequence on the radiochemical stability of [<sup>18</sup>F]7 in the final formulation. [<sup>18</sup>F]7 was indeed found to be more sensitive to radiolysis than other PET tracers routinely synthesized in our production center, such as [<sup>18</sup>F]FDG, [<sup>18</sup>F]FDOPA or [<sup>18</sup>F]FTYR.<sup>49–51</sup> For instance, the formulation of 19 GBq of [<sup>18</sup>F]7 in a 5% EtOH saline solution promptly led to the radiolysis of the tracer into multiple, unidentified radiolabelled compounds. After 4 hours, the radiolytes amounted for almost 70 % of the total activity in the bulk (Figure 5, trace A). A 3 to 5% radiolysis was also observed in similar formulations containing as little as 3.7 - 5.5 GBq of [<sup>18</sup>F]7. Taking a 100 µL aliquot from any of the tested formulations at EOS and letting it age separately from the bulk resulted in a high and constant radiochemical purity (RCP) of [<sup>18</sup>F]7 in

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the aliquot. This confirmed that the observed degradation was caused by the radioactivity of the sample, and not only by its chemical composition. It also indicated that the total activity in the formulation (*i.e.* the dose rate inside the solution) impacts the radiolysis kinetics more than the activity concentration alone, as should be expected. As a result, dilution of the formulation alone could not completely solve the radiolysis problem, so we moved to a chemical stabilization of the tracer. Ascorbic acid (HAsc) is an efficient and popular additive for the stabilization of radiotracers at high activity; as summarized in Figure 5, different activity concentrations and formulation compositions were tested in order to optimize the long-term (> 6 hours) stability of [<sup>18</sup>F]7. 1 mg/mL of HAsc added to the formulation allowed [<sup>18</sup>F]7 to remain completely stable for almost one hour. Following that short stability period, however, the radiotracer underwent a fast and nearly quantitative radiolysis (Figure 5, trace B). An increase of the ascorbic acid concentration addition of a citrate buffer to increase the pH of the formulation led to much more stable RCP's.

**Figure 5.** Evolution over time of the RadioChemical Purity (RCP) of [<sup>18</sup>F]7, at different activity, stabilizer or buffer concentrations.





A short, one-hour period of complete stability was however still followed by a measurable RCP decrease (Figure 5, traces C and D). Those results brought to light a strong influence of the pH on the radiolysis profile and kinetics. The replacement of ascorbic acid by sodium ascorbate allowed the formulation of [<sup>18</sup>F]7 at nearly neutral pH (pH 6.75) and enabled a better stabilization of the radiotracer, while suppressing the need for a buffer. For instance, 5 mg/mL of sodium ascorbate in the bulk solution allowed the formulation of up to 37 GBq of [<sup>18</sup>F]7 with RCP's exceeding 97% over 6.5 hours (Figure 6).

**Figure 6.** UPLC traces showing the radiochemical purity (Top) and the chemical purity (Bottom) of  $[^{18}F]$ 7 in formulations containing 33 GBq of the radiotracer. The peak saturating the UV detector results from the presence of sodium ascorbate in the formulation.



The cause of the 2-3% radiolysis observed in the 33 - 37 GBq formulations was attributed to the formulation on the C18 cartridge, during which all the activity is concentrated in a few cubic millimeters or less. The extremely high dose rates the radiotracer is exposed to during this step causes inevitable radiolysis that is negligible at low activities but increases with the total formulated activity. Therefore, although we were able to produce 96 GBq of [<sup>18</sup>F]7 from 285 GBq of [<sup>18</sup>F]fluoride, the routine production of [<sup>18</sup>F]7 is currently limited to 37 GBq.

#### The TEMPO Issue

This radiosynthesis has an important feature, which is the use of TEMPO (Scheme 3), a free radical and radical scavenger whose genotoxic properties were pointed out in a 2013 *in vitro* toxicology study.<sup>52</sup> As the presence of TEMPO is crucial for the efficiency of the radiofluorination, its removal was ruled out. The presence of a toxic compound is a potentially major drawback for a synthesis with intended clinical applications, since the synthesis must be

designed as to deliver a radiotracer formulation compliant with current regulations on the matter. However, we hereby demonstrate our ability to provide, without any major consequence over the synthesis design or on the Quality Control (QC) procedures, a formulation with a TEMPO content 500 to 1000 times below the specification. To the best of our knowledge, the maximal level of TEMPO tolerated in drugs is not clearly defined. We therefore followed the EMA (European Medicines Agency) recommendation<sup>53</sup> that refers to the ICH-Q3C guideline<sup>54</sup> for the determination of the maximal level of genotoxic impurities in drugs (ICH = International Council for Harmonization). According to our interpretation of the ICH-Q3C guideline, the tolerated concentration of TEMPO is 5.8 ppm, for a maximal injection volume of 10 mL in a 50 kg person (limit concentration in a worst-case scenario, see SI for detailed argumentation).

A few precautions were therefore taken during the design of the automation sequence, so that the probability of any TEMPO contamination reaching the final formulation remained as low as possible. For that purpose, the synthesis cassette was virtually divided in two parts. The left part of the cassette, denoted M1 in Figure 4, is where the radiosynthesis takes place. From the fluoride-18 recovery from the cyclotron through the HPLC injection with syringe S1, M1 is in direct contact with the solution containing TEMPO. The right part of the cassette, denoted M2, is where the purified radiotracer is collected from the HPLC and formulated into an injectable solution. The automated sequence was designed so that no potentially TEMPO-contaminated liquid was allowed to flow from M1 to M2 during the whole synthesis. An adequate washing sequence of M1 after the HPLC injection also ensured that the pressure inlet (left side of M1) could not blow contaminated leftover droplets from M1 to M2 during a necessary line flushing in the formulation step. Assuming the efficiency of our automation design against TEMPO contamination, the only remaining possibility for contamination was inefficient HPLC

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purification. The HPLC column and mobile phase were therefore chosen so that [<sup>18</sup>F]7 was eluted *before* TEMPO, in order to avoid any TEMPO contamination due to potential peak tailing. These two simple precautions proved to be effective, as the residual concentration of TEMPO in the formulations remained 500 to 1000 times below the 5.8 ppm specification (See Figure 7 and SI). Another less carefully developed sequence involved the HPLC injection of the crude *and* the formulation of the purified compound with the same syringe (S2), featuring a simple ethanol and saline wash of S2 between the two operations. As expected, that automated sequence resulted in slightly higher and more scattered contamination with TEMPO ( $40\pm25$  ppb, n = 5) in the formulations. The contamination nevertheless remained comfortably below the 5.8 ppm limit, demonstrating the efficiency of simple lines and syringe washes to almost quantitatively eliminate TEMPO from the cassette (Figure 7). This synthesis may therefore also be implemented on synthesizers that are less flexible in terms of cassette design.

Scheme 3. TEMPO free radical and its oxydized and reduced forms TEMPO+ and TEMPO-H.



**Figure 7.** Residual TEMPO-H in [<sup>18</sup>F]**7** bulk formulations using syringe S1 or S2 for the HPLC injection of the crude radiolabelling product (see also Figure 4), as determined by LC-MS analysis.



#### **Easy GC-FID detection of TEMPO**

The problematic of residual TEMPO in drugs has already been the subject of several publications, and its peculiar red-ox properties are well known.<sup>55–58</sup> In the presence of sodium ascorbate, TEMPO is quantitatively reduced into its TEMPO-H form, which suppresses its UV absorption band and excludes an otherwise very convenient UV detection.

Although we were very confident in the ability of our automation sequence to provide virtually TEMPO-free [<sup>18</sup>F]7 formulations (Figure 7), the very nature of TEMPO's toxicity imposed the development of a routine test for a possible excessive TEMPO contamination. The use of LC-MS is a fast and efficient way to accurately quantify TEMPO in our case. However, such equipment is not available at every production center, and it adds one more step to the routine QC. That is why we developed a GC-FID method for the routine compliance assessment of our formulations of [<sup>18</sup>F]7. The developed method allowed reaching Limits of Detection (LOD) and of Quantification (LOQ) that were well below the regulatory maximal concentration of 5.8 ppm: LOD = 0.6 ppm, LOQ = 2 ppm with RSD's < 5% (See Figure 8 and SI). The very low probability of significant contamination with TEMPO in routine batches was an ideal context to implement a Limit Test for the conformity assessment of the injectables.<sup>59</sup>

**Figure 8.** The implemented Limit Test for the assessment of the formulation's compliance towards TEMPO(-H) contamination features the GC-FID spectrum of a matrix solution spiked with 5.8 ppm of TEMPO, and that of an injectable from an automated production of  $[^{18}F]$ 7. The spectra are zoomed in on the relevant area for TEMPO-H detection. Result of the test: compliant.

Routine check for TEMPO-H contamination: raw GC-FID spectra from a Limit Test 0.8 0.6 TEMPO-H 0.4 g 0.2 8.0 0.6 0.4 0.2 3.5 3.6 3.8 3.9 3.7 min <sup>18</sup>F]7 injectable formulation

Routine QC (quality control) & GMP compliance

Standard (5.8 ppm TEMPO in matrix)

The quality control of the obtained [<sup>18</sup>F]7 formulation is constituted of classic radio- and UV-UPLC identification, (radio-)chemical purity assessment and specific activity measurement of the labelled compound (see SI). The extremely low level of cold impurities in the formulations (indicated by their UPLC analysis, see Figure 6) further demonstrated the efficiency of the automated HPLC purification of [<sup>18</sup>F]7. The QC of the injectable includes radionuclide identification, radionuclidic purity measurement, GC-FID Limit Test (for residual solvents and TEMPO), osmolality, pH, bacterial endotoxin testing, and sterility testing, as for any other cGMP drug. The detail of those quality control tests shall not be exposed here, as they are common and employed in every cGMP process. Besides the extensive and routine post-production QC, the rest of the radiosynthesis must also remain under proper control. The use of

single-use manifolds and of certified USP/Ph. Eur. reagents and solvents, the extensive characterization of the precursor and the determination of its shelf life are some factors that help guaranteeing the reproducibility and conformity of the radiosynthesis.

#### **PET Imaging**

**Figure 9. Top:** representative images of  $[^{18}F]$ 7 microPET acquisitions. Left panel, image obtained with the synthesis from this work. Right panel, image obtained with the former synthesis. **Bottom:** Time activity curves (expressed as SUV, mean ± SEM) of  $[^{18}F]$ 7 from this work (black symbols, n = 5) and from the former synthesis (open symbols, n = 5). Thal: thalamus, Cb: cerebellum.



The formulation of [<sup>18</sup>F]7 resulting from this radiosynthesis (5 mg/mL NaAsc in saline, no buffer) differs from the formulation that was generated with the former synthesis (see Scheme 1).

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The former formulation did not contain any stabilizer (the low activities produced did not impose any stabilization) and contained a citrate buffer. In order to assess that the difference in formulation or in cold impurity profile did not negatively impact the quality of the PET images, we performed test PET scans in rodents that allowed us to compare the two [<sup>18</sup>F]7 formulations.

The results presented in Figure 9 clearly show that [<sup>18</sup>F]7, regardless of the formulation composition, displayed high brain uptake and yielded distribution patterns that were highly consistent with previous work.<sup>4</sup> The time activity curves (TACs) extracted from thalamus and cerebellum were very close between the "new" and the "old" synthesis.

#### CONCLUSION

Primarily focused on the practicality of the developed precursor synthesis and automated radiosynthesis, this work covers all relevant aspects of the high-activity and cGMP production of up to 37 GBq of [<sup>18</sup>F]7. With an entirely new synthetic approach involving the one-step radiolabelling of a pyridyl(aryl)iodonium salt, our method ensures more than doubling the formerly possible production yield (Scheme 1) while shortening its duration from 120 to 50 minutes. We also demonstrate for the first time the ability of an iodonium salt to undergo efficient [<sup>18</sup>F]radiofluorination at high activity (up to 285 GBq) without any decrease of the radiochemical yield. Although the production of more than 37 GBq of [<sup>18</sup>F]7 is challenged by the sensitivity of this particular tracer towards radiolysis, it still attests that iodonium salts, with their usually remarkable stability, are more than ever promising precursors for applied late-stage aromatic radiofluorination of other key PET tracers.

In the context of the necessity to develop a simple and practical automated radiosynthesis, all the materials and apparatus involved in our production method were chosen for both their efficiency and their standard usage in routine production of other common PET tracers. We also developed a simple QC Limit Test using GC-FID detection in response to the issue raised by the use of genotoxic TEMPO. In addition, we showed that the residual TEMPO concentrations in the injectable could be kept 500 to 1000 times below the specification by simply using a thoughtful and logical design of the automated sequence. This production method will soon replace the former synthetic strategy (Scheme 1) for the production of [<sup>18</sup>F]7 for human use in our research center. The first human PET images acquired thanks to this method shall be published elsewhere.

In this work, multiple chemical, physical and analytical challenges were overcome in order to make an iodonium-based synthesis concept viable in the context of automated, routine productions in a cGMP environment. By deliberately exposing all encountered weak and strong points of iodonium salts in that respect, we provide key solutions that will enable other scientific teams to develop their own efficient productions of PET tracers of interest for human use using iodonium-based methods. More specifically, through providing PET centers with a simple, one-step and highly efficient synthetic method of a SV2A-specific radiotracer (Table 2), we trust that this work will significantly promote clinical research on most widespread neurological conditions, such as epilepsy and Alzheimer's disease.

**Table 2.** Contribution of this work to the synthesis of relevant SV2A-specific PET tracers. Data extracted from<sup>1,13,14</sup>.



\*unspecified if decay corrected or not

#### EXPERIMENTAL SECTION

#### **Materials & Methods**

Solvents and chemicals were purchased from VWR and Sigma Aldrich and used without further purification, unless otherwise stated. TEMPO was purchased as "purified by sublimation" grade. Racemic compound (*R*,*S*)-**4a** was purchased from UOrsY sales EU. Cold reference [<sup>19</sup>F]**7** and **4a** were provided by UCB Pharma (Belgium). Methyl-3-aminoisonicatinate **1** was purchased from KeyOrganics (UK). NMR spectra were acquired on a 400 MHz Bruker spectrometer and processed with MNova 10. The <sup>1</sup>H-NMR cGMP quantification method of **6** was developed on a 500 MHz Bruker spectrometer and maleic acid was used as a reference. The experimental details will be published elsewhere. An IBA Cyclone 18/9 cyclotron was used to produce <sup>18</sup>F-fluoride through the <sup>18</sup>O(p,n)<sup>18</sup>F reaction in a Niobium target containing 3.5 mL of <sup>18</sup>O-enriched water from Rotem Industries Ltd. The synthesis of [<sup>18</sup>F]**7** was automated on a Trasis All-In-One synthesizer, and a Phenomenex Luna C18 Column (5  $\mu$ m, 10 x 250 mm) was used for the semi-prep purification of the radiotracer. UV and radio chromatograms were recorded on a Waters® Acquity UPLC, using a BEH C18 Column (1.7  $\mu$ m, 2.1 x 50 mm) and an Eberline NaI gamma

detector. The chiral HPLC analyses for the measurement of the radiotracer's ee were carried out with an analytical Daicel Chiralpak® AD-RH column, using a 35/65 MeCN/pH 8 phosphate buffer as eluet at 0.3 mL/min. Mass spectra were acquired on a ESI-MS Waters® TQD coupled with a Waters® H-Class UPLC system. High-resolution mass spectra were acquired on a Bruker SolariX ESI-FT-ICR mass spectrometer. Gas Chromatograms were acquired on a ThermoScientific Trace 1310 gas chromatograph fitted with a PAL autosampler, using a 20meter Restek stabilwax crossbond carbowax PEG column (0.18 mm ID, 0.18 um df) and FID detection. Extensive information on the GC injection parameters is provided in the Supporting Information document. Melting points were measured on a Büchi B-545 device. The CHNS analysis of compound 6 was performed twice on an Intersciences Flash EA 1112 apparatus, and the related weightings made on a Mettler Toledo MX5 microbalance. The Xray diffractogram of compound 6 was recorded on a Bruker APPEX-II CCD diffractometer. Extensive data and information on the X-ray diffractogram acquisition are provided in the Supporting Information document, and the CIF file was deposited with Cambridge Crystallographic Data Centre (n°1480740). For the sake of graphical consistence and legibility, raw data sets from HPLC and GC detectors were plotted using Igor Pro. Raw data was not submitted to any transformation.

#### Chemistry

#### Methyl 3-iodoisonicotinate (2)

Procedure adapted from <sup>47</sup>. To a round-bottom flask (500 mL) were added acetonitrile (120 mL), methyl 3-aminoisonicotinate **1** (5 g, 33 mmol) and a magnetic stirrer. The stirred mixture was cooled down to -10 °C. Trifluoromethanesulfonic acid (11.7 mL, 132 mmol, 4 eq.) was then slowly added, using a glass syringe fitted with an inox needle. A solution of water (12 mL) containing sodium nitrite (4.78 g, 69 mmol, 2.1 eq.) and potassium iodide (14.24 g, 85 mmol, 2.6

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eq.) was then added dropwise (1.5 mL/min) to the reaction medium. The mixture was then removed from the cooling bath, and stirred for 1h15 at room temperature. The reaction was monitored by TLC (80/20 ethyl acetate/hexanes). Water (300 mL) was added to the reaction mixture, and solid sodium hydrogenocarbonate was then added until a slightly basic pH was reached. The opaque brown solution became clear-orange upon addition of a sat. solution of sodium thiosulfate (30 mL). The resulting solution was extracted with cyclohexane (6 x 100 mL), which is a selective solvent for the desired compound. The combined organic fractions were dried over magnesium sulfate and evaporated under reduced pressure to yield and orange oil that crystallizes quickly upon cooling. The obtained beige solid was redissolved in a minimum of cyclohexane and evaporated under reduced pressure to yield the desired compound as beige crystals (needles) (7.3 g, 28 mmol, 85 %). Melting point and <sup>1</sup>H NMR spectrum were consistent with literature data.<sup>60</sup>

<sup>1</sup>H NMR (400 MHz, Chloroform-*d*) δ 9.11 (s, 1H), 8.62 (d, *J* = 4.9 Hz, 1H), 7.64 (d, *J* = 4.9 Hz, 1H), 3.97 (s, 3H).

<sup>13</sup>C NMR (101 MHz, Chloroform-*d*) δ 165.48, 159.75, 149.21, 142.17, 124.60, 92.61, 53.12.

Melting point: 43 - 44 °C (cyclohexane)

ESI-MS: Observed m/z = 264 (M+1) Calculated: 263.95

#### **3-Iodoisonicotinaldehyde (3)**

To a dry round-bottom flask (100 mL) were added a magnetic stirrer, dry toluene (45 mL, distilled over sodium) and **2** (2.5 g, 9.5 mmol). The reaction mixture was cooled down to -96 °C (Dichloromethane/liquid nitrogen cooling bath) and a 1 M solution of di-isobutylaluminium hydride in toluene was added dropwise at a rate of 1.5 mL/min (19 mL, 19 mmol, 1.75 eq). The reaction mixture was allowed to stir 15 min at -96 °C after the addition was completed. The

reaction was then quenched (still at -96 °C) by the slow addition of methanol (5 mL). The first drops were added carefully, as the quenching caused a substantial foaming of the mixture. More methanol was then added (45 mL), and the reaction medium was removed from the cooling bath and allowed to stir at RT for 2 h. The crude mixture was poured into a beaker and placed on a 0 °C cooling bath. 4 M HCl (25 mL) was slowly added into the solution under vigorous magnetic stirring. After the addition was completed, the beaker was removed from the cooling bath and allowed to stir vigorously at RT for 1 h. Distilled water (30 mL) was added, and a concentrated solution of  $K_2CO_3$  was subsequently poured into the mixture until a basic pH was reached in the aqueous phase. The resulting mixture was extracted with ethyl acetate (3 x 80 mL), and the combined organic layers were dried over magnesium sulfate and evaporated under reduced pressure. The obtained crude yellow oil was redissolved in a 5 mL 50/50 mixture of ethyl acetate/hexanes and purified by flash chromatography on silicagel (80/20 ethyl acetate/hexanes) to yield the desired compound (2.03 g, 8.7 mmol, 93 %, pale yellow solid). <sup>1</sup>H NMR analysis was consistent with literature data.<sup>61</sup>

<sup>1</sup>H NMR (400 MHz, Chloroform-*d*) δ 10.05 (s, 1H), 9.11 (s, 1H), 8.68 (d, *J* = 4.9 Hz, 1H), 7.67 (d, *J* = 4.8 Hz, 1H).

<sup>13</sup>C NMR (101 MHz, Chloroform-*d*) δ 194.60, 159.60, 150.16, 140.77, 123.13, 97.23.

Melting point: 74 – 75 °C

HRMS: meas. m/z = 233.94068 (M+H) Predict. : 233.94104

#### (R)-Ethyl-4-amino-3-(3,4,5-trifluorophenyl)butanoate hydrochloride (4b)

In a dry, three-necked round-bottom flask were placed a magnetic stirrer, dry ethanol (50 mL, distilled over CaH<sub>2</sub>) and (R)-4-(3,4,5-trifluorophenyl)pyrrolidin-2-one (compound **4a**, 2.25 g, 10.4 mmol). The solution was cooled down and kept at 0 °C on an ice bath, and an excess of dry,

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gaseous HCl (generated by the slow addition of 100 g H<sub>2</sub>SO<sub>4</sub> on 100 g NaCl) was bubbled through the solution over one hour. The flask was then fitted with a condenser and the mixture was refluxed overnight at 105 °C. The solution was again cooled down to 0 °C prior to the dropwise addition of SOCl<sub>2</sub> (7.5 mL, 103 mmol, 10 eq.). The reaction mixture was allowed to stir at RT for 2 more hours. The resulting solution was filtered on a sintered glass filter and concentrated under vacuum, until a beige solid was obtained. The title compound was further vacuum-dried overnight and stored in the refrigerator (2.91 g, 9.8 mmol, 95%).

<sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>) δ 7.97 (s, 2H), 7.35 (dd, *J* = 6.6, 9.2 Hz, 1H), 4.04 – 3.91 (m, 1H), 3.41 (tt, *J* = 5.5, 9.8 Hz, 1H), 3.16 – 2.96 (m, 1H), 2.93 – 2.65 (m, 1H).

<sup>13</sup>C NMR (101 MHz, DMSO- $d_6$ )  $\delta$  170.51, 150.14 (ddd, J = 3.8, 9.6, 246.7 Hz), 137.90 (dt, J = 15.4, 247.2 Hz), 137.29 (td, J = 4.4, 7.2 Hz), 113.35 – 112.86 (m), 60.05, 42.79, 37.18, 13.94. ESI-MS: meas. m/z = 262. Predict. m/z = 262.11

#### (*R*)-1-((3-Iodopyridin-4-yl)methyl)-4-(3,4,5-trifluorophenyl)pyrrolidin-2-one (5)

To a dry round-bottom flask (100 mL) were added a magnetic stirrer, aldehyde **3** (2.35 g, 10.1 mmol), (R)-ethyl-4-amino-3-(3,4,5-trifluorophenyl)butanoate hydrochloride (compound **4b**, 3 g, 10.1 mmol, 1 eq.) and dry ethanol (45 mL, distilled over calcium hydride). The flask was fitted with a septum, flushed with Argon (or N<sub>2</sub>) for 5 minutes and then stirred at RT during the dropwise addition of triethylamine (1.77 mL, 13.1 mmol, 1.3 eq), and further stirred at RT for 2 hours (dark orange solution). The reaction medium was then cooled down to 0 °C, and sodium cyanoborohydride (764 mg, 12.1 mmol, 1.2 eq) was added to the reaction flask. The mixture was removed from the bath and stirred for 1 hour at RT. The mixture was again cooled down to 0 °C prior to the dropwise addition of a freshly prepared solution of sodium methoxide in ethanol. For that purpose, a clean piece of sodium (580 mg, 25.3 mmol, 2.5 eq) was dissolved in a solution of

dry methanol (1.5 mL, 37 mmol) in dry ethanol (10 mL). When the addition of sodium methoxide to the reaction flask was completed, the solution was further stirred at 0 °C for 1.5 h. The reaction was then quenched by the addition of a saturated aqueous ammonium chloride solution (50 mL) and vigorously stirred for 10 minutes. The resulting suspension was extracted with ethyl acetate (3 x 80 mL). The combined organic layers were dried over MgSO<sub>4</sub> and concentrated under reduced pressure. The obtained crude red oil was purified by silicagel chromatography (ethyl acetate/hexanes/triethylamine 80/20/0.5), to yield a colorless oil (2.5 g, 5.8mmol, 57%), that quickly became slightly yellow upon contact with air. For long-term storage, the oil was kept at -25 °C [**Important note:** when starting from racemic compound (*R*,*S*)-**4b** instead of **4b**, the obtained colorless oil could be crystallized: the oil was dissolved in a minimal amount of a 95/5 CH<sub>2</sub>Cl<sub>2</sub>/MeOH mixture, and diethylether (10 times the volume of CH<sub>2</sub>Cl<sub>2</sub>/MeOH used) was then added to the solution. The desired (*R*,*S*)-**4b** then crystallised upon ultrasound trituration or resting of the solution. The obtained off-white solid was washed with cold ether and dried under vacuum. Melting point: 132.5 – 134 °C].

NMR: <sup>1</sup>H NMR (400 MHz, Chloroform-*d*) δ 8.92 (s, 1H), 8.49 (d, *J* = 5.0 Hz, 1H), 7.15 (d, *J* = 4.9 Hz, 1H), 6.83 (dd, *J* = 6.2, 8.0 Hz, 2H), 4.55 (dd, *J* = 15.9, 71.3 Hz, 2H), 3.58 (p, *J* = 8.2 Hz, 1H), 3.52 (ddd, *J* = 6.8, 9.8, 165.8 Hz, 2H), 2.73 (ddd, *J* = 8.6, 17.0, 138.1 Hz, 2H).

<sup>13</sup>C NMR (101 MHz, Chloroform-*d*) δ 173.31, 157.85, 151.39 (ddd, *J* = 4.1, 10.0, 251.1 Hz), 149.48, 147.22, 138.20 (td, *J* = 15.1, 251.5 Hz), 137.99 (td, *J* = 4.7, 7.0 Hz), 123.69, 111.22 – 110.80 (m), 97.89, 53.76, 50.43, 37.94.

HRMS: meas. m/z = 433.00150 (M+H) Predict. : 433.00192

(R)-(4-Methoxyphenyl)(4-((2-oxo-4-(3,4,5-trifluorophenyl)pyrrolidin-1-

yl)methyl)pyridin-3-yl)iodonium trifluoromethanesulfonate (6)

In a dry round-bottom flask (50 mL) were placed a magnetic stirrer and a solution of compound 5 (1.1 g, 2.5 mmol) in dichloromethane (15 mL, dried over 4 Å molecular sieves). The solution was cooled down to -20 °C on an ice/NaCl bath, and trifluoromethanesulfonic acid (0.9 mL, 10.2 mmol, 4 eq.) was added dropwise. After 10 min of stirring, *m*-CPBA (purified by 3 pH-controlled extractions, followed by a recrystallization in dichloromethane and the vacuumdrying the obtained crystals, according to a reported procedure<sup>62</sup>) (1.1 g, 6.4 mmol, 2.5 eq.) was added and the mixture was further stirred at room temperature for 3 h, yielding a sticky, beige solid. The stirring may be restrained by the formed solid as the reaction proceeds. It is better, although not crucial, to release the magnetic stirrer from time to time with a glass rod. The flask was then cooled down to 0 °C and water (91 µL, 5 mmol, 2 eq.) was added to the reaction medium. The reaction flask was thoroughly shaken by hand, in order to efficiently quench the excess triflic acid. The mixture was allowed to stir for 10 more minutes at 0 °C. A solution of anisole (0.413 mL, 3.8 mmol, 1.5 eq.) in dichloromethane (5 mL) was then added dropwise to the reaction medium and the vial was stirred at RT for 1 h, during which the beige solid turned into a green oil that is the crude, protonated iodonium salt. The solvent was then evaporated under reduced pressure, and diethylether (30 mL) was added to the obtained crude compound. The green oil spontaneously crystallized into an off-white solid. The supernatant ether phase was removed, and the solid washed with Et<sub>2</sub>O (2 x 30 mL). The obtained protonated pyridyl(4methoxyphenyl)iodonium salt (white solid) was dried under vacuum, and deprotonated on a basic alumina plug according to a reported procedure<sup>42</sup>. Crystallization: the obtained solution of pure and deprotonated iodonium was evaporated until it became a pale, yellow oil. Dichloromethane was added while gently swirling the flask by hand (~15-20 mL). Diethylether was then added slowly (20 mL) with a Pasteur pipette, and was stopped before any persistent

trouble appeared (if any). The round-bottom flask was then gently swirled under ultrasound trituration for a few minutes (the precipitation of the iodonium compound **6** began after 30-60 seconds under ultrasound trituration). The obtained title compound was filtered, washed with fresh  $Et_2O$  and dried under vacuum (white "fluffy" powder, 1.28 g, 1.9 mmol, 75%).

<sup>1</sup>H NMR (400 MHz, Acetonitrile- $d_3$ )  $\delta$  8.96 (s, 1H), 8.73 (d, J = 4.9 Hz, 1H), 8.08 (d, J = 9.1 Hz, 2H), 7.57 (d, J = 4.9 Hz, 1H), 7.06 (d, J = 9.1 Hz, 2H), 7.02 (dd, J = 6.8, 9.0 Hz, 2H), 4.65 (dd, J = 15.5, 48.9 Hz, 2H), 3.84 (s, 3H), 3.62 (p, J = 8.5 Hz, 1H), 3.58 (dt, J = 7.9, 9.7, 153.8 Hz, 2H), 2.58 (ddd, J = 9.0, 17.0, 114.4 Hz, 2H).

<sup>13</sup>C NMR (101 MHz, Acetonitrile-*d*<sub>3</sub>) δ 175.81, 164.39, 155.32, 154.50, 151.90 (ddd, *J* = 4.0, 9.8, 247.3 Hz), 147.17, 139.94 (td, *J* = 4.5, 7.5 Hz), 139.36 (td, *J* = 15.0, 247.9 Hz), 139.05, 127.87, 122.02 (q, *J* = 320.9 Hz), 119.46, 119.09, 112.71 – 112.37 (m), 102.96, 56.72, 55.20, 50.08, 38.62, 37.89.

COSY, HSQC and HMBC NMR spectra: see SI.

Melting point: 145.8 – 146 °C (decomposition)

HRMS: meas. m/z = 539.04436 (M - OTf + H) Predict. : 539.04379

CHNS analysis (measured/predicted (%)): C (41.87/41.87), H(2.59/2.78), N(4.23/4.07), S(4.43/4.66).

X-ray crystallography: complies with expected structure. See extensive data in SI.

#### **Radiochemistry / Automation**

The enriched water containing <sup>18</sup>F-fluoride was directly recovered from the cyclotron target onto the Trasis All-In-One synthesizer and passed through a Waters® Sep Pak Carbonate Plus Light (46 mg of sorbent). The <sup>18</sup>F-fluoride was eluted in the reactor with 600  $\mu$ L of a 90/10 MeOH/Water eluent containing 10 mg K<sub>222</sub> and 0.6 mg K<sub>2</sub>CO<sub>3</sub>. The reactor was heated up to 125

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°C and the eluent was evaporated under a stream of nitrogen for 4.5 minutes. The pressurized precursor and TEMPO solution (in 1 mL MeCN) was then transferred to the reactor, which was sealed using built-in pinch valves to compress and close the in and out tubings of the reactor and heated to 125 °C for 10 min (labelling). The crude mixture containing the labeled compound was diluted with acetonitrile (0.5 mL) and saline (7 mL) and injected in a 9 mL HPLC loop for the semi-prep purification using a 62/38 H<sub>2</sub>O/MeCN mixture containing 0.1% of triethylamine as a mobile phase. [<sup>18</sup>F]7 was collected (retention time = 17.5-18.5 min) directly onto the manifold, in a vial that contains 5 mL of a 15 mg/mL sodium ascorbate solution for the stabilization of the labeled compound. The collected compound was further diluted (total volume = 40 mL) and passed through a Waters® Sep Pak C18 Plus Light cartridge for reformulation in two steps. [<sup>18</sup>F]7 was eluted from the cartridge with ethanol (700  $\mu$ L) into the final formulation flask. The injectable bulk formulation is constituted of 30 mL of a 0.9% NaCl solution, 5 mg/mL sodium ascorbate, 2.3 % ethanol and the radiotracer.

#### **PET imaging**

All animal experiments were performed according to the Helsinki declaration and conducted in accordance with the European guidelines for care of laboratory animals (2010/63/EU). All procedures were reviewed and approved by the Institutional Animal Care and Use Ethics Committee of the University of Liège, Belgium (LA1610005). Animals were housed in individually ventilated cages (2 per cages, room maintained at 22 °C and at humidity of approximately 40%, 12-h day-night cycle). 10 male Sprague-Dawley rats (Janvier, France) were used for all experiments, their mean body weight  $\pm$  SD at PET testing was 527  $\pm$  59 g. Standard pellet food and tap water were provided ad libidum.

The rats were first placed in an anesthesia induction box with a mixture of 4% isoflurane gas and air oxygen delivered at a flowrate of 1 L/min. After catheterization of a caudal vein, animals were placed prone in a dedicated bed (Minerve, France) and maintained under anesthesia during the entire micro- PET scan protocol with 1 to 2% isoflurane delivered via a cone mask. Respiration rate and rectal temperature were permanently measured using a physiological monitoring system (Minerve, France). Temperature was maintained at  $37 \pm 0.5$  °C using an air warming system. MicroPET scans were acquired on a Siemens FOCUS 120 microPET (Siemens, Knoxville, TN). PET data acquisition started with a 10-min 57Co transmission scan, with single event acquisition mode, following which [<sup>18</sup>F]7 "new" or [<sup>18</sup>F]7 "old" was injected in the lateral tail vein. Mean injected activity was  $45.2 \pm 1.3$  MBg and  $42.2 \pm 5.3$  MBg, respectively. Emission data were then recorded in list mode for a total of 60 min. Data were reframed as follow: 6 x 5 s, 6 x 10 s, 3 x 20 s, 5 x 30 s, 5 x 60 s, 8 x 150 s, 6 x 300 s. PMOD software version 3.6 (PMOD Technologies Ltd., Zurich, Switzerland) was used to process the imaging data and to extract time activity curves (TACs) in volume of interests (VOIs) positioned over the thalamus and cerebellum of PET images. These VOIs were obtained using PMOD rat brain atlas. Individual TACs were normalized by the injected dose and the animal weight, and then expressed in standard uptake value (SUV).

ASSOCIATED CONTENT

**Supporting Information**. Additional figures and data sets complementing stereochemistry assurance, labelling regioselectivity, SA determination, description of the cassette used for the automated synthesis, derivation of ICH guidelines for TEMPO, UPLC-MS determination of TEMPO, GC-FID determination of TEMPO, NMR spectra of compounds 1 - 6 and extended

crystallographic data of **6**. This material is available free of charge via the Internet at http://pubs.acs.org.

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#### **Author Contributions**

All authors have given approval to the final version of the manuscript.

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#### ABBREVIATIONS

AIO, All-In-One<sup>®</sup>; cGMP, current good manufacturing practices; DIBAL, diisopropylaluminium hydride; EOS, end of synthesis; ESI, electro-spray ionization; FDG, fluoro-deoxyglucose; FDOPA, fluoro-DOPA; FTYR, fluoro-L-tyrosine; FID, flame ionization detector; GC, gas chromatography; GMP, good manufacturing practices; PET, positron emission

tomography; HRMS, high resolution mass spectrometry; ICH, international council for harmonization; LOD, limit of detection; LOQ, limit of quantification; *m*-CPBA, *m*chloroperoxybenzoic acid; MS, mass spectrometry; NMR, Nuclear Magnetic Resonance (CPD, composite pulse decoupling; APT, attached proton test; COSY, correlation spectroscopy; HSQC, heteronuclear single quantum coherence spectroscopy; HMBC, heteronucear multiple bond correlation); MS, mass spectroscopy; ORTEP, Oak Ridge thermal ellipsoid plot; Ph. Eur., European pharmacopoeia; QA, quality assurance; QC, quality control; RCP, radio chemical purity; RCY radio chemical yield; RT, room temperature; RSD, relative standard deviation; SOS, start of synthesis; SUV, standard uptake value; SV2A, synaptic vesicle glycoprotein 2A; TAC, time-activity curve; TEMPO, (2,2,6,6-Tetramethylpiperidin-1-yl)oxy; TLC, thin layer chromatography; USP, United States pharmacopoeia; VOI, volume of interest.

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