Digital autoradiography for efficient functional imaging without anesthesia in experimental animals: Reversing phencyclidine-induced functional alterations using clozapine

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\textbf{A B S T R A C T}

Autoradiography (ARG) is a high-resolution imaging method for localization of radiolabeled biomarkers in ex vivo specimen. ARG using 2-deoxy-\textit{o}-glucose (2-DG) method is used in to study drug actions on brain functional activity, as it provides results comparable to clinically used functional positron-emission tomography (PET). The requirement of slow analog detection methods and emerging advances in small animal PET imaging have, however, reduced the interest in ARG. In contrast to ARG, experimental animals need to be restrained or sedated/anesthetized for PET imaging, which strongly influence functional activity and thus complicate the interpretation of the results. Digital direct particle-counting ARG systems have gained attraction during the last decade to overcome the caveats of conventional ARG methods. Here we demonstrate that the well-established 2-DG imaging method can be adapted into use with contemporary digital detectors. This method readily and rapidly captures the characteristic effects of phencyclidine (5 mg/kg, i.p.), a dissociative agent targeting the NMDAR (N-methyl-\textit{o}-aspartate receptor), on regional glucose utilization in the adult mouse brain. Pretreatment with antipsychotic drug clozapine (6 mg/kg, i.p.) essentially abolishes these effects of phencyclidine on brain functional activity. Digital ARG produces viable data for the regional analysis of functional activity in a fraction of time required for film development. These results support the use of digital ARG in preclinical drug research, where high throughput and response linearity are preferred and use of sedation/anesthesia has to be avoided.

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\textbf{1. Introduction}

Glucose is the predominant energy substrate of the brain under normal conditions and is required for all neuronal functions (Sokolo et al., 1977). The correlation between glucose utilization and functional activity of neurons has been determined to the extent that the glucose consumed by a single action potential can be quantified (Sokolo, 1999). Clinical symptoms of various psychiatric disorders, such as schizophrenia (Mitelman et al., 2018; Cleghorn et al., 1989; Farkas et al., 1984; Kishimoto et al., 1987), depression (Drevets et al., 1997, 1992; Biver et al., 1994; Baxter et al., 1989), and bipolar disorder (Drevets et al., 1997; Hosokawa et al., 2009; Keener and Phillips, 2007; Brooks and Vizueta, 2014) are reflected in distinctive patterns of altered cerebral glucose utilization that can be restored using pharmacological treatments (Buchbaum et al., 1992; Bartlett et al., 1998; Wolkin et al., 1996; De Crescenzo et al., 2017; Kang et al., 2012; Forlenza et al., 2014; Little et al., 2005). In preclinical research, this enables the use of functional imaging methodology to screen for potential drug candidates by their ability to modulate disorder-related functional alterations.

The 2-deoxy-\textit{o}-glucose (2-DG) method developed by Sokoloff et al. (1977) measures the rates of energy metabolism in discrete brain regions in both normal and altered states using the correlation between glucose utilization and functional activity. In this method, radiolabeled (usually \textsuperscript{14}C or \textsuperscript{3}H) glucose derivate 2-DG is systemically injected into an animal, followed by an uptake period ranging generally from 5 to 45 min (Sokoloff et al., 1977; Duncan et al., 1989). During the uptake period, the compound is transported and trapped inside cells in a quantity directly relative to their ongoing metabolic (i.e. functional) activity. When the brain is sectioned, the distribution of the radiolabel can be detected ex vivo to form a spatial visualization of regional glucose utilization during the uptake period. The 2-DG method was originally developed to use with autoradiography (ARG), which is a sensitive, high resolution nuclear imaging technique used to study distribution of radiolabeled compounds. While the method has since

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been adapted for use in vivo and clinical setting with 18F-labeled 2-DG (FDG) and positron emission tomography (PET), ARG still enables higher spatial resolution (Koba et al., 2011; Yang et al., 2016; d’ArCY and Sundwall, 2000; Solon, 2015; Schmidt and Smith, 2005; Barthe et al., 2012), cheaper costs (Fine et al., 2014; Upham and Englert, 2003), and longer temporal window for imaging (Solon and Kraus, 2001). Due to ARG imaging being performed using dissected ex vivo samples, it also does not require the use of anesthesia or physical restraint during imaging, both of which are known to strongly alter functional brain activity and connectivity, glucose utilization, and several other neurobiological measures (Ohata et al., 1981; Ori et al., 1986; Herkenham, 1981; Kohtala et al., 2016; Theilman et al., 2019; Borelius et al., 2013; Hildebrandt et al., 2008; Momosaki et al., 2004; Toyama et al., 2004; Eintrei et al., 1999; Lenz et al., 1998; Paasonen et al., 2018; Antila et al., 2017). Thus, the small animal PET and other small animal imaging methods often have significant confounding factors interfering with research of treatments for central nervous system (CNS) disorders.

There is an enormous need for new and more efficient tools in the field of CNS drug development, where the amount of novel discoveries has dwindled over the recent decades (Caraci et al., 2017; Preskorn, 2011). With the advances in direct particle-counting systems, the new digital ARG devices have surpassed the conventional film and phosphor screen-based ARG in linearity (Kelly and McCulloch, 1983; Lear, 1986; Ott et al., 2000; Cabello et al., 2007), dynamic range (Upham and Englert, 2003; Barthe et al., 1999, 2004), and sensitivity (Nicole Barthe et al., 2012; Ott et al., 2000; Solon and Moyer, 2014), reducing the acquisition times from months to just few hours. In this study, we assessed the potential of using digital ARG with gas-based charged-particle imager as a screening method for preclinical research by quantifying the ability of an antipsychotic drug clozapine in restoring the functional alterations induced by phencyclidine (PCP), a psychotomimetic that acutely triggers behavioral and perceptual changes resembling schizophrenia or psychosis (Javitt et al., 2012). The used PCP model has been previously validated using conventional ARG (Duncan et al., 1998, 2000; Duncan et al., 2003; Weissman et al., 1987), but the long exposure times and the use of rats have reduced viability of the method for cheap high-throughput screening, where short imaging times and cheaper laboratory rodents would be preferred. Our aim was to establish whether this type of imaging could be utilized for rapid screening of functional effects produced by pharmacological agents.

2. Materials and methods

2.1. Animals

All animal experiments were carried out according to the National Institute of Health (NIH) guidelines for the care and use of laboratory animals and approved by National Animal Experiment Board of Finland (License number ESAVI-2015-2269). A total of 15 healthy male C57BL/6 J mice (Charles River Laboratories, Kisselegg, Germany), aged 7–8 weeks and weighing 23.5 ± 0.76 grams were used in the experiment. Animals were group-housed (4 animals in a cage) in stainless steel cages and kept on a controlled 12-h light/dark cycle at an ambient temperature of 22 ± 1 °C with access to food and water available ad libitum. Three hours prior to the experimental procedures, cages housing the animals were moved to experiment room and the access to food was suspended to ensure stable blood glucose level and adequate radiolabel uptake. Experiments were conducted between 3 and 8 h after light onset.

2.2. Drugs and administration

An aqueous solution of 3H-2-DG (2-[1,2-3H (N)]-deoxy-D-glucose, 20 Ci/mmol; PerkinElmer Inc., Boston, USA) was administered in a volume of 100 μl via i.v. injection to tail vein. Phencyclidine (PCP, 1-(1-}

Phenyln cyclohexyl)piperidine hydrochloride; Tocris Bioscience, Bristol, UK) was dissolved in sterile saline at a concentration of 1 mg/ml. Clozapine (CLZ; Sigma-Aldrich Co. LLC, St. Louis, USA) was dissolved in 0.1 M hydrochloric acid, adjusted to pH 7 with 0.1 M sodium hydroxide, and then diluted to a concentration of 1.2 mg/ml with sterile saline. Both PCP and CLZ injections were administered via i.p. injections in volume of 5 ml/kg, resulting in doses of 5 mg/kg and 6 mg/kg, respectively. Vehicle (VEH), sterile 0.9% saline solution, was injected i.p. in a same volume as the drugs.

The animals were divided to three treatment groups: 1) VEH + PCP, 2) CLZ + PCP, and 3) VEH + VEH (n = 5 in all groups). At first, the mice received i.p. injection of either CLZ or VEH and were transferred back to their home cages. At 30 min after the first injection, either PCP or VEH was administered and the mice were transferred to a temporary empty cage. 15 min after the PCP or VEH injection the mice were restrained and received 100 μl of 3H-2-DG in a concentration of 375 μCi/ml, or approximately 1.6 μCi/g body weight, via an intravenous injection to tail vein. The animals were terminated after a glucose uptake period of 15 min post-radiotracer injection. A schematic for experimental timeline is presented in Fig. 1.

2.3. Sample processing

The animals were euthanized exactly 15 min after 3H-2-DG injection using CO2 and a terminal blood sample was collected via cardiac puncture. The blood samples were centrifuged (2000 g, 10 min, +4 °C) and the resulting supernatant collected for analysis. The brains were immediately dissected and flash frozen by immersing them in 2-methylbutane (isopentane) pre-chilled on dry ice for under a minute (Gonzales-Lima, 1992; Maker and Lehrer, 1971). No fixative was used in order to ensure propagation of β-particles from the tissue to detector. Dissected brains were then stored at −80 °C until moved to −20 °C the day before sectioning. Twenty (20) μm thick coronal sections were thaw mounted on a plain glass slides in a cryostat (Leica CM1950) at −14 °C every 100 μm beginning approximately from bregma +3,55 mm and ending approximately to bregma −7,55 mm, resulting in 112 coronal sections of each brain.

2.4. The autoradiographic analysis of 3H-2-DG uptake

Autoradiographs were acquired using a gaseous scintillator-coupled β-emission detecting charge-coupled device (CCD) camera, Betalmager Racer (Biospace Lab, Paris, France), using a 6-h acquisition time and a full-field zoom setting (250 × 200 mm). 12 pre-chosen regions of interest (ROI) were outlined using Paxinos’ and Allen’s mouse brain atlas overlays in an image processing software M3 Vision (Biospace Lab, Paris, France) (Lein et al., 2007; Paxinos and Franklin, 2012). Each ROI was analyzed on 8–15 brain sections, depending on the size of the structure. The regional functional activity was analyzed with a semi-quantitative method (Vyzayovskiy et al., 2004; Duncan and Stumpf, 1991), which allows the study of more time-limited effects of drugs compared to the original method requiring 45-min radiolabel uptake period and multiple blood samples. The detected rates of regional beta-emission (cpm/mm²) were normalized to an internal control (corpus callosum) to account for biological variation, and thus the data are expressed as the ratio of cpm/mm² of a ROI to that of corpus callosum. Comparison of treatment effects to all regions and individual ROI of

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**Fig. 1. Study timeline for drug and radiolabel administration.**
treatment groups were performed using one-way analysis of variance (ANOVA) followed by a Tukey’s post-hoc test.

2.5. Plasma radioactivity analysis

A tritium concentration standard was prepared by creating a proportional 12-step serial dilution with a dilution factor of 1:2, using 375 µCi/ml ³H-2-DG and rat plasma. A liquid scintillation counter (MicroBeta2 Plate Counter, PerkinElmer) was used to measure the activity (cpm) of all standard solutions and animal terminal plasma samples. The plasma radioactivity (µCi/ml) content of each animal at the time of termination was then calculated by fitting the measured sample activity to the standard. The total circulating blood and plasma volumes were estimated using literature (Diehl et al., 2001). The data was analyzed using one-way ANOVA, followed by a Tukey’s post-hoc test.

3. Results

3.1. Behavior

Experimenters followed behavioral responses of the animals after treatment administration and documented the visual observations. The mice displayed expected acute behavioral responses to the drug treatments. Based on visual observations, PCP induced increased locomotor activity and stereotypy (Chen et al., 1965; Chen et al., 1959). The stimulating properties of PCP were also visibly observed in the cardiovascular system (vasoconstriction of tail vein when injecting radiolabel, increased heart and breathing rate). Mice injected with CLZ became significantly sedated within five minutes from injection (Coward, 1992). With the current doses, the observed effects of CLZ completely overwhelmed those of PCP in animals subjected to both treatments. These responses to the drugs persisted until the termination of the animals.

3.2. The autoradiographic analysis of ³H-2-DG uptake

Full-field autoradiographic acquisition using Betalmager ™Racer resulted in digital images with an initial pixel resolution of 1488 × 1190, which equal to a pixel size of 168 µm² on a 250 mm × 200 mm field of view. Representative sections with annotated ROIs (region-of-interests), shown as greyscale and heat map images, are shown with their respective atlas overlays in Fig. 2.

PCP treatment induced pronounced alterations in the 2-DG uptake pattern throughout the whole brain (Fig. 3). The increase in glucose uptake was found to be neuroanatomically selective, with some regions responding to PCP significantly more than others. Overall, when comparing the ROI median values of studied brain regions, the mice treated with PCP had significantly higher regional functional activity than control (Fig. 4). The regional functional activity of the PCP-treated animals without CLZ pretreatment was significantly higher in comparison to control group in all the individual measured ROI, except for cerebellum and inferior colliculus. CLZ pretreatment altered the functional activity of the PCP-injected mice in majority of the ROI. CLZ pretreatment failed to restore the PCP-induced increase in functional activity only in the amygdala.

3.3. Plasma analysis

Calculated mean radioactivity in the plasma at the time of termination was 0.227 (± 0.079) µCi, suggesting that only roughly 1% of the original injected radiolabel dose remained in the blood circulation. Activity difference between VEH + VEH and VEH + PCP groups was found non-significant, but the activity of CLZ + PCP group surpassed that of other groups (p ≤ .01). The results of plasma analysis are shown in Supplementary Fig. S1.

4. Discussion

While functional imaging using 2-DG method has been extensively described using conventional (film) ARG, the present study is the first (to our knowledge) to demonstrate functional imaging using newer direct particle-counting system. Digital ARG enabled the use of low activity radiolabel tritium and acquisition time of only 6 h to produce enough data for quantification. While piloting the method, we exposed additional sections on phosphor image plates (IP) for 13 days and scanned them using FLA scanner on 10 µm resolution (Supplementary Fig. S2). Due to the low specific activity of ³H-2-DG, the IP detection produced quantification-impairing amount of background noise and low contrast between brain regions, demonstrating the superior sensitivity and dynamic range of the digital detector.

Functional imaging using ARG is comparable to human PET imaging, widely used in clinical setting, and therefore possesses high translational value. The method described in this study has the same underlying rationale as the semi-quantitative functional autoradiography method, making the acquired results comparable with previous literature based on traditional analog autoradiography (G. E. Duncan et al., 1993; Duncan et al., 1999; Miyamoto et al., 2000). Indeed, the selective metabolic activation seen in the present study bears resemblance to NMDA antagonist-induced alterations seen in humans and in animal models (Vollenweider et al., 1997a; Vollenweider et al., 1997b; Långsjö et al., 2004; Gao et al., 1993). We selected the dose of PCP based on the report by Xue-Min Gao et al. (1993) who also described prominent metabolic effects and behavioral phenotypes resembling the positive symptoms of schizophrenia. It should be noted that with this dose range PCP triggers also non-NMDA receptor-mediated effects, namely dopaminergic actions (D. C. Javitt and Zukin, 1991). However, the observed patterns in metabolic activation more closely mirrored those produced by other NMDA receptor antagonists, rather than pure dopaminergic psychoactive drugs such as amphetamine (Miyamoto et al., 2000).

The plasma radioactivity analysis was used to control that all animals were properly injected with radiolabel. CLZ administration (and resulting sedation) was reflected as higher remaining radiolabel in the circulation compared to other groups. In addition, plasma analysis revealed one outlier in the VEH + PCP group, where the animal had slightly delayed radiolabel administration time, resulting in higher amount of radiolabel remaining in circulation at time of termination. However, as the quantification of ROI glucose utilization was normalized to the internal standard of each animal, this did not impact the overall analysis of functional activity.

High sensitivity of the direct particle-counting systems enables detection of even minute amounts of radionuclide present in the samples. This establishes the two greatest benefits of the method, reduction in time it takes to conduct functional imaging studies and the possibility to use low-energy isotopes. Decreasing the acquisition time from months or weeks to only hours enables high throughput and considerably streamlines experiments utilizing functional autoradiography, thus lowering costs and speeding up research. Additionally, the digital acquisition can be followed in real time, while the conventional methods require estimation and piloting of the exposure time based on used amount of radionuclide. Use of low-energy radiolabels brings forth further practical benefits. In comparison to ¹⁸F used in FDG PET, isotopes such as ³H or ¹⁴C are considerably cheaper and enable working under consignment limits issued by European Union in Council Directive 2013/59/Euratom (2013) and United States Code of Federal Regulations 49 CFR § 173.436 (2011). These benefits make digital ARG fast, cheap, and safe method for functional imaging suitable for screening treatment effects on brain activity. By enabling rapid assessment of functional effect of treatments, digital detectors possess
significant advantages in academic and industrial use over conventional autoradiography.

As a drawback, the high sensitivity contributes to the visually noisy appearance of the autoradiographs as seen in Fig. 2. Therefore, the visual appearance of the autoradiographs acquired using direct particle-counting systems is clearly inferior to conventional film autoradiographs. However, it should be noted that the visual noise does not affect image quantification, as the quantification is not done using the image itself, but accurate numeric temporal and spatial coordinates of each detected β-particle emission stored by the system. Compared to conventional autoradiography, there is also no loss of information in quantification resulting from the analog-to-digital conversion of the scanned film or phosphor plates. Nevertheless, the current method has considerably lower spatial resolution in comparison to conventional autoradiography, where even cellular resolution can be achieved. Therefore, if the goal is to acquire more accurate spatial localization data of functional activation (e.g. activation of individual nuclei), alternative digital detector types or conventional autoradiography should be considered. Basic comparison of conventional autoradiography, digital autoradiography, and small animal PET imaging is provided in Table 1. The spatial resolution achieved using the current gas-based charged-particle imager was found to be sufficient for detecting the functional activity in discrete regions of mouse brain, enabling rapid screening process. It can also be argued that in pharmacological experiments, response linearity for dose-effect quantification should be prioritized over spatial resolution, especially if the goal of the experiment is to quickly screen different compounds.

Obvious limitation of the method is that it only allows determination of glucose utilization at one time point. For FDG PET, newer
approaches have been developed to enable measurement of dynamic alterations in brain activity during FDG PET imaging in humans (Villien et al., 2014; Hahn et al., 2016; Rischka et al., 2018). Alternative metabolism-reflecting radiolabels, such as $^{15}$O, can also be utilized to assess dynamic changes of limited temporal nature (Sheppard et al., 1983; Carter et al., 1997). Longitudinal studies with $^{15}$O have been demonstrated feasible in small animals (Wehrl et al., 2014). However, as discussed in this manuscript, most preclinical methods utilizing PET detection are impeded by the requirement of anesthesia or restraint. While some novel methodology have been utilized for functional PET and SPECT imaging of freely moving animals, these methods have lower spatial resolution and lack the decades of validation behind functional ARG (Baba et al., 2013; Spangler-Bickell et al., 2016).

As the goal of this research was methodological development, only male mice were used in order to increase the comparability to previous research. Significant majority of previous research into PCP-induced hyperfrontality has utilized male rodents (Kargieman et al., 2007; Duncan et al., 1998; Gao et al., 1993; Weissman et al., 1987). However, female rodents have been shown to metabolize PCP slower in comparison to males (Shelnutt et al., 1999). While more research is required to validate the used pharmacological model in both sexes, this factor does not affect the primary focus of this study, which was development of novel methodology for digital autoradiography.

5. Conclusion

The method described in this study provides a compelling alternative to small animal PET and SPECT imaging, as it can be performed...
on samples collected from freely moving animals. As discussed, the use of anesthesia or restraining of the animal during imaging are both major confounding factors impairing the translational value of results acquired with PET and SPECT imaging. Thus, ARG remains as the cheapest, highest resolution, and most clinically translatable functional imaging method for preclinical studies, especially in CNS research. Overall, our results support the use of digital autoradiography-based functional imaging for screening the effects of drugs on brain activity.

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### Disclosures
The authors declare that there is no conflict of interest in the present study.

### Ethical statement
All animal experiments were carried out according to the National Institute of Health (NIH) guidelines for the care and use of laboratory animals and approved by National Animal Experiment Board of Finland (License number ESAVI-2015-2269).

### Declaration of Competing Interest
None.

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### Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.pnpbp.2020.109887.

### Table 1
General comparison of conventional autoradiography, digital autoradiography, and small animal PET imaging methodology (N Barthe et al., 2004; Miller, 2018; Goertzen et al., 2012; Yamane et al., 1995; Upham and Englert, 2003).

| Conventional autoradiography | Digital autoradiography | Small animal PET |
|------------------------------|--------------------------|------------------|
| + Can study effects of drugs on brain activation without anesthesia | + Can study effects of drugs on brain activation without anesthesia | + Can be used to study dynamic changes |
| + Extremely high spatial resolution (Film: < 1 μm, phosphor image plates: ~50 μm) | + High spatial resolution (~20–50 μm) | + Same animal can be imaged multiple times |
| + Possible to use low-energy radiolabels | + High sensitivity | + Three-dimensional analysis |
| + Low costs | + Possible to use low-energy radiolabels | |
| + Old and validated method | + Dual labeling possible | |
| - One time point measurement | + Real time measurement | |
| - Long exposure times | - One time point measurement | |
| - Low sensitivity | - Expensive detector | |
| - Low dynamic range | | |

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