The Use of HiSPECT to Investigate Dopaminergic Involvement in the Development of Stereotypic Behaviour

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Summary

Functional molecular imaging is becoming increasingly popular for *in vivo* research on small animals, because it has a number of scientific advantages over *ex vivo* methods. The molecular parameters themselves can be used in other areas of investigation also, such as monitoring of the dopaminergic and serotonergic involvement in the development of stereotypic behaviour. In Single Photon Emission Computed Tomography (SPECT), a radioactive substance with specific affinity for a certain molecular target is injected intravenously and after a period of time the radioactivity that is not washed out from the region of interest, is measured. A relative measure of quantification, i.e., the binding index (BI), can then be calculated. This paper aims to introduce a broad readership to one possible application of SPECT by presenting preliminary data about the dopamine transporter (DAT) status in the Mongolian gerbil (*Meriones unguiculatus*). ^{99m}Technetium-labelled Ethylcysteinate Dimer tracer (24.42 ± 5.92 MBq) was injected in the femoral vein of four gerbils to provide brain perfusion images that allow anatomical identification of DAT-rich regions that were imaged in another four gerbils using ^{123}I-labelled FP-CIT tracer (44.33 ± 11.66 MBq). Furthermore, the optimal scan time for FP-CIT was established in one gerbil. The study was successful in obtaining brain perfusion images as well as demonstrating regional binding of FP-CIT to the basal ganglia, DAT-rich areas in the brain. The optimal scan time for DAT-imaging was 4 ½ hours. Our preliminary data suggest the Mongolian gerbil is a suitable model for combining SPECT and behavioural observations in the investigation of stereotypic behaviour.

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Introduction

Experiments to study gene function, identify (patho-)physiological processes, or investigate receptor abundance or pharmacokinetics in drug development generally use *ex vivo* small animal models (*Beekman & van der Have, 2007*). As a result, data collection is usually limited to cross-sectional studies since animals need to be sacrificed at the time of data collection. In cases where the animal does not need to be sacrificed, only a small number or limited amount of sample(s) can be taken from one animal. For instance, blood sampling volume is limited to 10% of the circulating blood volume for single sampling whereas this is only 1% for repeated samplings every 24 h (*Morton et al., 1993*). Consequently, if longitudinal studies

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involving repeated measurements are required, large numbers of animals are needed (*Peremans et al., 2005*) and/or the interval between samplings should be sufficiently long. Adhering to the idea of reduction in the use of experimental animals (*Russell, 2005*) which is a part of the 3R-idea currently governing legislation and research protocols, it is evident that *in vivo* alternatives to study molecular dynamics are gaining interest, especially since animals can serve as their own control.

One such alternative concerns functional imaging modalities (positron emission tomography, PET and single photon emission computed tomography, SPECT), where an injected radioactive ligand binds to a specific target (receptor, transporter or enzyme) and allows *in vivo* imaging of biochemical molecules with minimally invasive procedures (*Peremans et al., 2005*). The differences between PET and SPECT are discussed in depth elsewhere (*Peremans et al., 2003; Wirrwar et al., 2001*). Briefly, and aside from the fact that the type of radiation in both is different, β+ radiation in PET and γ-emission in SPECT, the radioligands created for SPECT have a longer half-life than those for PET, meaning that long-distance transport is possible and on-site production via sophisticated and expensive cyclotrons is no longer needed. Since the different types of application are limited only by the availability of suitable radioligands, SPECT is more accessible because tracers are increasingly commercially available as well as reasonably priced.

SPECT can be used to monitor a number of parameters. First, cerebral blood flow can be measured through SPECT as the activity of neurons is coupled to cerebral blood flow by the oxygen/glucose needs in their metabolism (*Kuschinsky, 1993*). Other than providing information about brain activation patterns, the perfusion images also indicate which are intra- and extra-cerebral structures and therefore provide a framework of reference for anatomical identification of other molecular targets (*Nikolaus et al., 2005b; Perez-Garcia et al., 2003*). Second, brain receptors or transporters can be evaluated *in vivo*. From a behavioural point of view, the most investigated molecules belong to the dopaminergic and serotonergic systems (D1 receptor (*Arnt, 1985; Cabib et al., 1998; McBride & Hemmings, 2005*); D2 receptor (*Cabib et al., 1998; Nikolaus et al., 2005a; Presti et al., 2004*); Dopamine Transporter (*Lehmann et al., 2002; Nikolaus et al., 2005b; Pogorelov et al., 2005*), Serotonin-2A receptor (*Eison & Wright, 1992; Giegling et al., 2006; Peremans et al., 2003; Schiller et al., 2003*), Serotonin Transporter (*Chen & Lawrence, 2003; Kugaya et al., 2003*)). SPECT allows for indirect semiquantification of such a target molecule, in that a binding index (BI) is calculated. This number is a ratio of the activity (counts/pixel) in the area of interest and that of a reference region - where the molecule under investigation has been presumed or demonstrated to be absent (*Booij et al., 2002*). The radioactivity present in the area of interest consists of radiation from the tracer bound specifically to the molecule of interest or aspecifically to non-target molecules as well as radiation from free, unbound tracer. Because the reference region does not contain the target molecule, the radiation found there is only composed of the latter two. As a result, once equilibrium is reached between specifically bound, aspecifically bound and free tracer in both regions, changes in the BI reflect altered abundance of the target molecule.

One of the major concerns when considering the use of SPECT in research is the radiation exposure involved (*Talbot & Laruelle, 2002*), both for researchers and experimental animals. However, from a radiation worker's point of view, European Directives (European Council Directive 96/29/EURATOM, 13th May 1996) and subsequent national legislation carefully govern radioprotective measures. Data about the radiation burden on experimental animals is scarce (*Peremans et al.* 2005), but some authors provide parameters in an abstracted animal model to get an idea of the radiation burden for different isotopes

(*Funk et al., 2004*). From this model, which reduces animals to elliptoid forms, it was concluded that the smaller the animal is, the larger the organ dose will be, potentially changing physiological properties of tissue as a result of organ damage, and the recommendation was made to not let radiation dosage exceed what is needed for acceptable resolution.

Publications which provide examples of how to apply SPECT in small animal research, either to support other data from other scientific areas or for the molecular data itself, are usually confined to neuromedical or medical imaging journals. Therefore, the goal of this paper is to acquaint a broad readership with the use of SPECT as a powerful and minimally invasive tool for monitoring biological processes *in vivo*. More specifically, while investigating feasibility in the Mongolian gerbil, we provide a detailed methodological description of the SPECT procedure and present data about cerebral blood flow (perfusion) and the dopamine transporter (DAT) status. It is our intention in future studies to use DAT as a physiological marker, complementing behavioural observations, for dopaminergic involvement in the ontogeny of stereotypic behaviour of these animals.

Materials and Methods

Prior to the experiment, approval for the procedures from the Ethical Committee of the Faculty of Veterinary Medicine at Ghent University was obtained. The Laboratory for Ethology is licensed for breeding and carrying out experimental procedures on gerbils (LA2400377; LA1400096).

Animals

Mongolian gerbils (*Meriones unguiculatus*) aged 378 ± 63 days were used in this study. Our subjects were bred from four pairs of Mongolian gerbils purchased from the outbred SPF RjTub:MON stock from Elevage Janvier (Le Genest-St-Isle, France). The animals were group-housed (2-3 animals per cage) in Makrolon type IV cages (55 x 33 x 20 cm, Bio-Services, Schaijk, The Netherlands) in the colony room at the department of Animal Nutrition, Genetics, Breeding and Ethology. The cages were bedded with Gold Mix wood shavings (Carfil, Turnhout, Belgium) and cage enrichment was provided in the form of Mini-Tork™ paper tissue (Tork, Guildford, Australia), hay, and chew blocks. The chew blocks were homemade by cutting and dividing branches from apple and cherry trees. Pellets of 2016 Teklad Global 16 % protein rodent diet (Harlan, Horst, The Netherlands) and tap water were available *ad libitum*. All animals were kept under a 14:10 light:dark cycle with lights off between 9:00 and 19:00. Room temperature averaged 20 ± 1 °C whereas relative humidity values ranged between 30 % and 60 %. Continuous ventilation was provided.

Nine animals were included: one was used to determine the optimal DAT acquisition time point, an additional four to determine the DAT distribution

Figure 1. Overview of gamma camera. Three camera heads with pinhole collimation rotate around the animal in the scanning bed (white arrow) in 10 angular steps of 36°.

Figure 2. Pinhole collimator used in small animal SPECT.

at that optimal time, and finally perfusion images from four gerbils provided an anatomical frame or reference. Images of the DAT distribution were obtained with ^{123}I-FP-CIT tracer, DaTSCAN (GE Healthcare, Buckinghamshire, England) whereas a conventional ^{99m}Technetium Ethylcysteinate Dimer tracer, Neurolite (Brystol-Myers Squibb, New York, USA) was used in the perfusion studies. Tracers were heated to gerbil body temperature (38°C; (*Mele* 1972)) and injected intravenously in the femoral vein as described by Moons et al. (2008). Briefly, the animals were exposed to 4% isoflurane in an induction chamber and as soon as they were immobilized, we transferred them to a restrainer to clip the fur on the hind leg and inject the radioligand using a 30G bent needle as the animal was regaining consciousness.

Subsequently, the animal was returned to its home cage, which was heated by an infrared lamp (200W). We opted to allow the gerbils to regain consciousness between injection of the tracer and image acquisition for two reasons: first, the time between the injection and the scan in perfusion versus DAT studies was different. As this would result in different lengths of anaesthesia, it was likely to cause different physiological responses (*Peremans et al., 2005*). Second, the interval between injection and scanning in DAT studies consisted of a few hours and keeping an animal anaesthetized for this length of time would jeopardize its recovery.

Prior to scanning, the animals were again placed in the induction box and anaesthetized with isoflurane (4% induction, 1.8% maintenance). Once anaesthetized, they were transferred to the scanning bed and placed in a sternal recumbency. To make sure the animal was always positioned exactly the same, a line was drawn on the scanning bed for positioning the gerbil's head using the ears as reference. A mouthpiece ensured continued administration of anaesthetic drug. Because the animals were prone to heat loss throughout the 20-minute scan, we adapted the scanning bed to include an apparatus based on circulating warm water (38°C) to promote post-anaesthesia recovery by minimizing body heat loss (*Moons et al., 2008*).

Acquisition

A micro-SPECT was performed using a conventional triple head gamma camera (Triad, Trionix, Twinsburg, OH, USA), adapted with 3 multi-pinhole collimators (6 holes, 2,5 mm ∅) (Bioscan, Washington, USA) as shown in figures 1 and 2. Data were acquired for 20 minutes in step-and-shoot mode (10 steps, 36° angular step, 120 sec per step) on a matrix of 256 x 256. This acquisition mode for a 3-detector system results in a radial sampling of 12 degrees.

Image processing

Multi-pinhole images performed on the Trionix gamma camera were transferred to the HiSPECT system (Impact Version 1.0, Bioscan Inc, USA). Images were reconstructed using a dedicated ordered subset-expectation maximisation (OSEM) algorithm (Scivis, Göttingen, Germany). Pixel size was 1.78 mm. The resolution obtained with HiSPECT is 2.0 mm (*Dobbeleir et al., 2007*). With the help of dedicated software from Hermes (Nuclear Diagnostics, Sweden), reconstructed data from DAT imaging were fused to the data obtained from the perfusion study. Irregular regions of interest (ROI) were manually drawn over the region of the basal ganglia and over the cerebellar area as reference region devoid of DAT (*Booij et al., 2002*).

The ratio of global uptake in the basal ganglia (average activity in left and right basal ganglia) to cerebellar activity was used as a relative measure of specific bindi

Results

Perfusion

A perfusion study was performed in four animals to provide an anatomical frame of reference for the images containing DAT binding activity. Animals were scanned 15 minutes after injection of 24.42 ± 5.92 MBq ^{99m}Technetium Ethylcysteinate Dimer tracer. Figure 3 represents a reconstructed horizontal perfusion image of the cerebral and spinal structures of the animal alongside a plastinated brain. This shows that the gerbil cerebellum and cortical structures can indeed be distinguished through perfusion tomographic images.

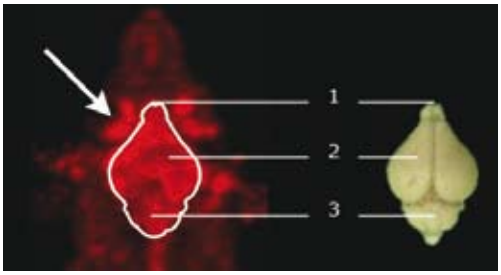

Figure 3. Dorsal view of a reconstructed gerbil brain perfusion image (left) and dorsal view of a gerbil brain that was fixated in formaldehyde (right). Structures of the brain which can be distinguished from the perfusion image are indicated. 1: olfactory bulb, 2: cerebrum, 3: cerebellum. The white arrow indicates high uptake of radioligand in the orbital area, possibly in the Harderian glands.

DAT

Assured that we could anatomically identify gerbil brain structures, we continued to perform DAT scans using 44.33 ± 11.66 MBq ^{123}I-FP-CIT tracer per animal (n = 4). In humans, the cortex/cerebellum binding ratio of ^{123}I-FP-CIT tracer is known to reach a plateau, a requirement for reliable calculation of the BI (Binding Index), around 3 hours post injection and remains stable for another three hours (*Booij et al.,* 1999). To confirm this in gerbils, we intended to perform a 20-minute scan every 30 minutes for 6 hours, starting immediately after injection. Because prolonged anaesthesia can pose survival risks to the animal and the fact that this part of the experiment was merely intended to confirm the species-specific binding optimum, only one animal was used for this purpose (n = 1). Unfortunately, because of hardware problems and the time it took to fix them, the first scan only started at 190 minutes after injection. Figure 4 denotes a graph of the cortex/cerebellum-binding ratio of ^{123}I-FP-CIT tracer to the DAT plotted against time after injection. Although we were unable to perform scans the first three hours after injection, the graph shows that in gerbils the tracer-binding index does not achieve a plateau before 4 ½ hours, which remains stable at 1.2. Specific binding to the DAT sites was observed by the regional distribution of the radioactivity in the brain, which correlated well with the known presence of DAT sites (basal ganglia, figure 5). Table 1 contains the binding index data performed at the scan optimum on four additional gerbils.

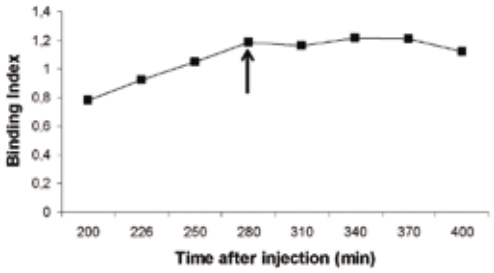

Figure 4. Binding index as a function of time after injection. A plateau is reached at 280 minutes (arrow), which is the middle point of the scan taken between 260 and 290 minutes.

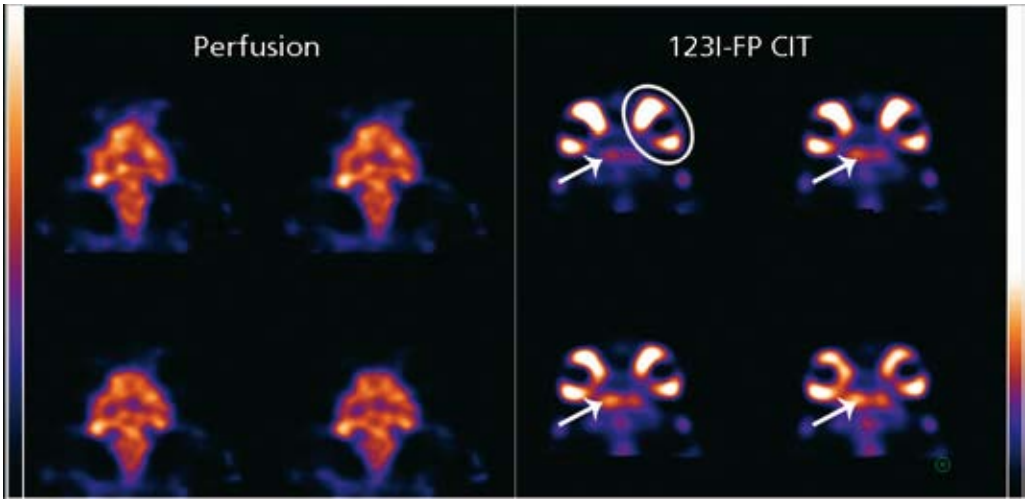

Figure 5. Images reconstructed from cerebral blood flow (left) and DAT scan (right). White arrows on the right indicate cores of ^{123}FP-CIT specific binding, corresponding to the location of the basal ganglia. High FP-CIT uptake is also present in the orbital area, possibly corresponding with the Harderian glands.

Table 1. Binding index (average of left and right basal ganglion acitivity per pixel / activity count in cerebellum) in four gerbils calculated from DAT-binding activity 4 ½ hours after tracer injection. Individual animals are denoted by capital letters.

	A	B	C	D
BI	1.56	1.60	1.65	1.64
μ ± SEM	1.61 ± 0.021			

Discussion

Our data represent the preparatory studies of an experiment to study the role of dopamine in the ontogeny of stereotypies in Mongolian gerbils. The aim was to investigate whether the SPECT-technique could successfully be applied, to identify potential pitfalls and to present the results to a broad readership of laboratory animal scientists to acquaint them with some of the possibilities of the system. Two significant pitfalls, i.e., intravenous injection and preservation of body heat during anaesthesia are discussed separately elsewhere *(Moons et al., 2008*) in order to describe in detail the procedural refinements that were applied. The use of anaesthetics can have an effect on several physiological parameters, and even tracer uptake, and it is advised that the anaesthetic procedure is identical throughout the entire study (*Peremans et al.* 2005). Nonetheless, isoflurane, when compared to injectable (*Tsai et al., 2007*; *Weinandy et al., 2005*) or other gaseous anaesthetics (*Toft et al,. 2006*) seems to have less impact on the physiology of an animal. Furthermore, only mild side effects on haemodynamics, e.g., microvascular tissue perfusion, have been identified (*Szczesny et al., 2004*). Also no changes in heart rate and mean arterial blood pressure were observed in anaesthetized mice that were observed for 4 hours. However, in that same study Szczęsny and coworkers found that arterial blood pressure did

decrease with increasing isoflurane concentration. Other researchers observed a decreased BI for the DAT using ^{125}I-PE21 when isoflurane was used as an anaesthetic. However, conditions were different from those in this study as the tracer was injected 3-4 h after induction of anaesthesia (*Elfving et al., 2003*). Since it is impossible to perform aSPECT scan in an animal that is only physically restrained, it is suggested that the depth of anaesthesia should be appropriate without unnecessary overdosing and that the same anaesthetic protocol should be used for all subjects in a study.

When looking at the results of the SPECT scans, we found that injected ^{99m}Technetium Ethylcysteinate Dimer tracer using the HiSPECT multipinhole system and processing software from Bioscan successfully produced images allowing the anatomical identification of brain structures in the Mongolian gerbil.

The images produced by ^{123}I-FP-CIT resulted in specific binding in areas corresponding to the location of the basal ganglia, which is in accordance with results from studies in other species (*Alvarez-Fischer et al., 2007; Nikolaus et al., 2005b*). In verifying the optimal time after injection of the tracer to perform the scan, our data showed –despite a delay in the start of the scan- that a plateau in the cortex/cerebellum binding index ratio is reached at 4 ½ hours. In other species such as mice and rats, data are usually acquired 2 hours after injection of the tracer (*Alvarez-Fischer et al.* 2007; *Booij et al.* 1997; *Nikolaus et al.* 2007). The binding index calculated in four gerbils was slightly higher than that of the animal used to determine the optimal scanning time. This particular animal was older (464 versus 371 days) and research in humans has shown that the abundance of DAT can decrease with age (*Volkow et al.* 1996).

Conclusion

These preliminary data suggest that SPECT is a workable tool for monitoring of the DAT status in the Mongolian gerbil. Perfusion studies using a conventional ^{99m}Tc tracer resulted in images suitable for anatomical identification of the areas of specific binding to DAT of the FP-CIT tracer.

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