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COMMENTARY

Additional methodological considerations regarding optimization of the dose of intracerebroventricular streptozotocin A response to: "Optimization of intracerebroventricular streptozotocin dose for the induction of neuroinflammation and memory impairments in rats" by Ghosh et al., Metab Brain Dis 2020 July 21

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Abstract

A recent article by Ghosh et al. entitled "Optimization of intracerebroventricular streptozotocin dose for the induction of neuroinflammation and memory impairments in rats" provides an important new set of information on neuroinflammation and cognitive deficit in a rat model of sporadic Alzheimer's disease (sAD) based on intracerebroventricular administration of streptozotocin (STZ-icv) in Charles-Foster rats in the early post-treatment period of 21 days. This comment is supposed to supplement the aforementioned manuscript by providing additional perspective on important factors that should be taken into account in the process of optimization of the streptozotocin (STZ) dose for intracerebroventricular treatment, and provides a brief overview of possible sources of variation of experimental results reported by different groups working with STZ-icv rodent models.

Dear editors,

We have read with great interest a recent publication entitled *Optimization of intracerebroventricular streptozotocin dose for the induction of neuroinflammation and memory impairments in rats* by Ghosh et al. (2020). The main topic of this article, elucidation of the optimal dose of streptozotocin for induction of memory impairment in rats, has been extensively studied in our laboratory ever since the introduction of the model by Hoyer and co-workers (Mayer et al. 1990), and our group represented by Lackovic and Salkovic (Lacković and Šalković 1990). In line with that, this comment is supposed to supplement the aforementioned manuscript by providing additional perspective on factors that should be taken into account in the process of optimization of the streptozotocin (STZ) dose for intracerebroventricular treatment.

Intracerebroventricular streptozotocin (STZ-icv) administration has become a widely used method for modelling neuroinflammation and neurodegenerative processes. Although the exact mechanism of action of STZ-icv is still not entirely clear, STZ-icv administration generates pathological features known not only to be the key players in the shared pathophysiology of the neurodegenerative disorders in general, like neuroinflammation, oxidative stress and mitochondrial dysfunction (Sharma and Gupta 2001; Kraska et al. 2012; Correia et al. 2013; Bloch et al. 2017; Biswas et al. 2018; Mishra et al. 2018), but additionally induces AD-specific hallmarks like cholinergic deficits and pathological accumulation of amyloid ß and hyperphosphorylated tau protein as well as the metabolic dysregulation in the brain (Salkovic-Petrisic et al. 2013; Kamat et al. 2016; Grieb 2016;). Amyloid pathology in STZ-icv model can be detected at different steps along the amyloid pathways, from the increased expression of the amyloid precursor protein (APP) and its pro-amyloidogenic

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cleaving enzyme (BACE1) in the early stages, to the development of cerebral amyloid angiopathy and the pathological accumulation of Aβ1–42 in the cortico-hippocampal region both intraneuronally and extracellularly, with the latter observed not earlier than several months after the STZ-icv treatment (Lester-Coll et al. 2006; Salkovic et al. 2011; Knezovic et al. 2015; Bloch et al. 2017; Mishra et al. 2018). Pathology in the homeostasis of tau protein in STZ-icv models has been seen as its hyperphosphorylation mostly at Ser199/202, Thr205 and Ser396 epitopes found in the cortico-hippocampal region from early to the advanced stages, and positive AT8 immunoreactivity corresponding to the early neurofibrillary changes (although not neurofibrillary tangles) found in hippocampus in the advanced stage (Lester-Coll et al. 2006; Barilar et al. 2015; Knezovic et al. 2015; Guo et al. 2017; Biswas et al. 2018; Mishra et al. 2018). Impaired cholinergic transmission in STZ-icv model has been evidenced most frequently by increased activity of acetylcholinesterase, the acetylcholine-degrading enzyme (Reeta et al. 2017; Biswas et al. 2018; Rodrigues et al. 2019;). Moreover, and most importantly, metabolic dysregulation in the brain, recognized as the pathophysiological core of AD (Hoyer 2004; de Felice et al. 2014; de la Monte and Tong 2014) has been found also as a prominent feature of the STZ-icv model. STZ-icv-induced significant, region-specific decreases in activities of enzymes involved in glucose metabolism, decrease in ATP concentrations and energy have been well documented (Hoyer and Lannert 2007; Barilar et al. 2020), and further supported by imaging studies demonstrating *in vivo* decreased glucose metabolism in the brain of STZ-icv-treated rats and monkeys (Heo et al. 2011; Knezovic et al. 2018). Impaired insulin signaling occurs in the brain as an early response to the central administration of diabetogenic compound like STZ, demonstrated by decreased expression of insulin1 mRNA and insulin receptor (IR) protein, accompanied by decreased phosphorylation of elements downstream IR signaling pathways, from insulin receptor substrate 1 (IRS1) to the glycogen synthase kinase 3 (GSK3) whose isoforms α and ß have been implicated in regulation of homeostasis of amyloid ß and phosphorylation of tau protein, respectively (Lester-Coll et al. 2006; Grünblatt et al. 2007; Barilar et al. 2015). Such a dysfunctional insulin signaling results in brain insulin resistance considered to be implicated in the etiopathogenesis of AD and closely related to cognitive decline (Hoyer 2004; de la Monte and Tong 2014; de Felice et al. 2014; Kellar and Craft 2020). This is further supported by promising results of testing the therapeutic potential of approved antidiabetic drugs, including the intranasal insulin therapy, in clinical and non-clinical trials in sAD patients and STZ-icv models as their counterparts (Talbot and Wang 2014; Guo et al. 2017; Boccardi et al. 2019; Pilipenko et al. 2020; Kellar and Craft 2020).

Therefore, a large body of evidence indicates that starting from the cognitive impairment as the most prominent feature, underlying neurochemical, metabolic and structural changes in STZ-icv-treated animals resemble those found in sporadic Alzheimer's disease (sAD) patients (Mosconi 2013; Sekoe and Hardy 2016; Dansokho and Heneka 2018; Gao et al. 2018; Hampel et al. 2018; Tobore 2019). Taken together, all indicate that STZ-icv model might provide insights into the onset and dynamics of some of the pathomechanisms related to the pathogenesis of sAD. In contrast, *a priori* manipulation of the specific genes in the transgenic animal models of AD offers limited insight in the temporal dynamics and the nature of the pathophysiological processes and thus provides a lesser translational value in comparison with the non-transgenic models like STZ-icv model.

A combination of promising findings on sAD-like changes in the STZ-icv model, the fact that STZ is affordable and the model relatively easy to establish, and increasing awareness that transgenic mice AD models are more suitable for elucidation of pathology related to familial rather than the sporadic form of the disease, have all encouraged the use of the STZ-icv model for both testing the potential therapeutics and understanding of the molecular mechanisms orchestrating the process of neurodegeneration (Salkovic-Petrisic et al. 2013).

As briefly reviewed by Ghosh et al. (2020), a number of variations of the original model are in use today with no clear agreement on either the optimal dose of STZ, or the time-point and type of cognitive test appropriate for the assessment of cognitive decline. For this reason, we acknowledge the effort by Ghosh et al. to contribute to a better understanding of the model by investigating the effect of 3 different doses on neuroinflammatory markers and cognitive function assessed by radial arm maze test in 5 time points within the early post-treatment period of 21 days (9, 12, 15, 18 and 21 days after the induction procedure). Attempts to assess time- and dose-responses to STZ-icv have already been reported in the literature e.g. (Kraska et al. 2012; Knezovic et al. 2015), but optimization of the model should take into account a number of other methodological finesses that are known to significantly affect both outcomes mentioned in the manuscript - neuroinflammation and memory impairments.

In the further text, we wish to supplement the aforementioned article through expanded scope on some additional methodological parameters closely related to the dose that should be taken into account during the process of optimization of the STZ-icv protocol, with the emphasis that the model should, as always, be adapted to best fit the specific experimental purpose.

Preparation of STZ and the choice of vehicle

The protocol used for the preparation of the STZ solution can greatly influence the outcome of the STZ-icv procedure. Vehicle treated animals are usually used as controls, so any *potential biological effects of different vehicles* should be anticipated and an additional untreated control group should be

introduced when appropriate. Saline, artificial CSF and citrate buffer are all standardly used, but differences are to be expected as, for example, local acidic environment induced by intracerebral administration of citrate buffer has been shown to potentiate the cellular uptake of oligodeoxynucleotides possibly by protonation of membrane proteins involved in the process (Zhou et al. 2019). One other study indicated that intracerebroventricular administration of just 20 nmol of citrate is able to reduce food intake and body weight, inhibit hypothalamic AMP-activated protein kinase, and potentiate glucose uptake and insulin signaling in the periphery (Stoppa et al. 2008). Since these results have been questioned by others (PubPeer report n.d.), the effects of citrate administration remain to be further explored. Furthermore, *selection of the vehicle determines the solubility of STZ*. In our laboratory, 0.05M citrate buffer (pH 4.5) is standardly used to ensure optimal dissolution, as it has been shown that an approximately 6 times greater amount of STZ can be dissolved in 0.01M citrate buffer in comparison with PBS (pH 7.2) (e.g. CaymanChemical product information for streptozotocin n.d.). Additionally, administration of a *fresh vs. previously frozen solution* could make a difference. This is critical, as, in our protocol, a fresh STZ solution is made right before intracerebroventricular administration and all animals in the STZ-icv group receive the treatment in a time window of 15 minutes as originally recommended for the STZ protocol for induction of diabetes by the National Institute of Diabetes and Digestive and Kidney Diseases Animal Models of Diabetic Complications Consortium (STZ protocol for induction of diabetes by the National Institute of Diabetes and Digestive and Kidney Diseases n.d.). Immediate administration following dissolution has originally been proposed due to the alleged instability of STZ; however, this has been clearly refuted as experimental evidence shows that STZ is stable in acidic buffer solution for several days at room temperature (Oles 1978). Also, t*he chemical structure of STZ itself* might influence the reactions of STZ. STZ usually exists as a mixture of α and β anomers, with α anomers being present in larger quantities (≥ 75%) upon dissolution and reaching the equimolar equilibrium with the β form in the first 90 minutes due to mutarotation of the glucopyranose ring (Oles 1978). It has been shown that α anomers are more toxic, and that freshly prepared STZ solution exerts more pronounced toxic effects in comparison to the anomer-equilibrated one (de la Garza-Rodea et al. 2010). Finally, even when mutarotation is taken into account, we have observed a batch-to-batch variation in potency of the toxin, so additional exogenous factors, outside the experimenter's control, should also be considered when discussing the optimal dose for the STZ-icv.

STZ-icv administration protocol

Specific STZ administration protocols for induction of cognitive deficits differ between laboratories, with two greatest methodological differences being related to the *intracerebral vs. intracerebroventricular* administration, and administration *with vs. without the stereotaxic apparatus*. In our laboratory, intracerebroventricular administration is standardly used; however, different groups reported cognitive deficits following intracerebral administration of the compound as well (Lester-Coll et al. 2006). Furthermore, our standard protocol for intracerebroventricular administration (described previously by Noble et al. (Noble et al. 1967)) does not include head fixation in the stereotaxic apparatus. Instead, a freshly made STZ solution is administered with a Hamilton microliter syringe with a custom made stopper by an experienced experimenter in order to maximally reduce the time of the procedure. The accuracy of the injection procedure was previously validated by injecting methylene blue dye. Although challenging to establish, we believe this protocol to be optimal in the case when a larger number of animals per group is needed as, in our experience, reduced variability due to uniform time of STZ administration, and diminished need for prolonged anaesthesia compensate for potential variability introduced by omitting the stereotaxic navigation. *Short vs. prolonged duration of STZ administration* following cannulation of the ventricle also greatly differs in literature. Interestingly, we have found rapid administration times as low as 10 s per administration still produce satisfactory results, although a significant amount of STZ solution is lost due to back flow following microinfusion. In contrast, some laboratories infuse STZ solution over several minutes, and leave the microcannula in place to ensure the whole dose has been administered. The exact reason why the STZ-icv model works even after rapid administration is perplexing; however, involvement of a hypersaturated mechanism or potentiating effect of ventricular distension following microinjection provide possible explanations. Finally, *administration of a bolus dose vs. split doses* could be responsible for the inconsistency in the results; some laboratories use a bolus dose in a single injection, while others use a method of splitting a total dose in 2–3 repeated injections protocol with 48 hours being the standard inter-treatment interval used. Similarly, *bi- vs. uni-ventricular administration* should be considered as a possible source of the results inconsistency; some laboratories use biventricular administration, while some administer the whole dose unilaterally (e.g. see Table 1 in (Salkovic-Petrisic and Hoyer 2007)), with *volumes of the administered solution* being one additional variable factor. We have found the method of repeated biventricular injection of 2 μl of STZ solution administered per ventricle to provide the best results. One additional factor to consider is the pharmacodynamic interaction with the type of anaesthesia used in the protocol, as some drugs might potentiate or alleviate the effect of STZ, however, this important interaction still remains to be explored. For example ketamine, a noncompetitive N-methyl-D-aspartate receptor antagonist that is often used as anesthetic in STZ-icv induction protocols, has been

suggested both as a toxin that can be used to model AD (Gilles and Ertlé 2000), and as a potential therapy (Smalheiser 2019).

The most appropriate behavioral tests

Cognitive dysfunction in the STZ-icv model has been confirmed using a wide variety of behavioral assessment tools in different time-points. However, due to the *behavioral complexity* of the model, and specific strengths and weaknesses of different behavioral tests used for cognitive assessment, the choice of the optimal test in STZ-icv is not straightforward, and should be carefully chosen based on the goal of the specific experiment. In their manuscript, Ghosh et al. (Ghosh et al. 2020) used a radial arm maze test to extract data on both working and reference memory errors. The use of "dryland" tests in the STZ-icv model is especially complicated by *excessive spontaneous locomotor activity* of rats treated with STZ. Interestingly, prominent locomotor hyperactivity has been reported in the first publication with behavioral examination of the STZ-icv model (Mayer et al. 1990), however its importance and implications for behavioral testing have been largely ignored. It is to be expected that shorter and more frequent *interaction* with objects, and greater overall *distance* and average *speed* will introduce serious bias in standard cognitive assessment protocols. For example, greater distance travelled inside the arena is likely to result in an increased number of erroneous entries, not just as a result of potential cognitive dysfunction, but also as a result of the increased number of total entries. Similarly, greater movement velocity inside the passive avoidance test is likely to result in decreased entrance delay (even during habituation), that should be taken into account. One way to minimize the potential error introduced by discrepant locomotor behavior, apart from modification of original protocols, is to use behavioral tests usually reported by means of proportional outcomes. One such example is the novel object recognition test (NOR) as interaction with the novel object is standardly normalized to total object interaction time. Nevertheless, it should be noted that NOR is also not completely immune to behavioral differences observed in STZ-icv animals, as STZ-icv rats tend to inspect the object more frequently and for shorter durations, another finding in concordance with behavioral observations reported in the original publication by Hoyer's group (Mayer et al. 1990). For this reason, a significant effort should be made to minimize this effect by *making necessary adjustments to the original behavioral protocols*, as well as to *construct appropriate statistical models* to control for possible locomotor (and other) sources of bias post-hoc. Apart from that, it should be taken into account that *different tests measure distinct cognitive functions* that probably deteriorate in the STZ-icv model at a different rate, and that the design of different behavioral batteries makes them variably susceptible to systematic bias arising from behavioral complexity of STZ-icv-treated animals. In conclusion, a timeline of STZ-icv-induced cognitive deficits should be made with caution with adequate attention directed to potential confounding factors, as different experimental designs are likely to suggest variable cognitive deterioration rates (and suggest variable doses of STZ to be optimal). Furthermore, as evidence suggests that multiple mechanisms are implicated in the orchestration of STZ-icv pathophysiological changes with different temporal patterns in regards to their most pronounced effects (early "neurotoxic" patterns and late AD-like pattern) behavioral changes should be interpreted and discussed in the context of their potential non-linear progression (Knezovic et al. 2015).

Animal- and pathology stage-related contributing factors

Finally, some factors not strictly related to the STZ-icv protocol could significantly affect the choice of the optimal dose of STZ. *Animal species and strain* has been shown to play an important role in pathophysiological changes in STZ-icv rats. For example, Bloch et al. reported that the development of STZ-icv-induced dementia is associated with obesity and peripheral metabolic abnormalities in Lewis rats (Bloch et al. 2017), a phenomenon not observed in the Wistar strain so far. Ghosh et al. determined the optimal dose of STZ in male albino Charles-Foster rats which have not been explored so far as a STZ-icv model, however it remains open for debate whether the same dose can be translated to other strains and species, considering some differences in brain anatomy, histology and physiology as well as underlying genetic and neurochemical diversity (Bart Ellenbroek 2016). *Sex and age* of animals at the time of STZ-icv are also probably important, although information on this is scarce. Nevertheless, data from the experiments on STZ-induced diabetes suggest a pronounced effect of age on STZ-induced toxicity (Wang-Fischer and Garyantes 2018). Finally, a dose-dependent biphasic pattern of changes in cognitive performance in STZ-icv treated Wistar rats should be taken into account (Knezovic et al. 2015). Considering an acute cognitive decline found up to 1 month post-treatment and a tendency of its normalization approximately 3 months after treatment (following administration of 0.3, 1 and 3 mg/kg), which is then followed by a slowly progressing cognitive decline up to 9 months (for 1 and 3 mg/kg, but not for 0.3 mg/kg dose), *a time after the STZ-icv treatment* at which testing of the cognitive performance is examined, has a great influence on the results. Since these follow-up studies showed that AD-like structural changes appear later on in the course of the disease indicating a slow linear progression, the post-treatment time is of utmost importance for optimization of the STZ-icv dose, depending on the

underlying pathology whose correlation with cognitive decline is being explored.

To conclude, the effort by Ghosh et al. is praiseworthy as there is a great need to systematize the knowledge on the STZ-icv model and provide uniform guidelines to minimize experimental differences arising from variable methodologies used by different groups. We consider this to be of critical importance, as good and reliable models for sAD are a prerequisite for elucidation of etiopathogenesis of the disease and the discovery of efficient therapies. Nevertheless, as numerous factors are crucial for a successful induction of the STZ-icv model and interpretation of the results, we believe individual optimization of the model to best fit experimental goals of different laboratories is still the best approach given that appropriate validation tests are conducted, and complete experimental data is made available for other researchers.

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Compliance with ethical standards

Conflict of interest None.

Ethics committee approval Not applicable.

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