Cognitive characterization of Long-Evans rats in a streptozotocin-induced Alzheimer's disease model

PhD thesis

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1. Introduction

Alzheimer's Disease

Alzheimer's disease (AD) is the most common neurodegenerative disease which makes up approximately 70% of all types of dementias. The disease affects approximately 50 million people worldwide and this number is estimated to grow to 120-150 million by 2050. Unfortunately, AD is relentlessly progressive and currently there is no known medical treatment that would cure the disease. The main histological hallmarks of the disease are senile plaques and neurofibrillary tangles (NFTs). The most common cognitive symptom is memory impairment. Progressive cell death of neurons leads to cerebral cortex shrinkage and enlarged ventricles.

Many hypotheses have been made about the pathomechanism of the illness, although the exact cause is still unknown. The amyloid cascade hypothesis suggests that amyloid-β (Aβ) generation and aggregation is the triggering factor of AD. The tau hypothesis is based on the fact, that tau is abnormally phosphorylated during the disease, leading to detachment of tau from the microtubule, formation of neurofibrillary tangles and neuronal degeneration.

Type 3 diabetes hypothesis

A more recent hypothesized cause is the insulin resistant brain state, present during AD, why it is also called – although misleadingly- Type 3 diabetes. Human postmortem studies show signs of insulin resistance during AD. It manifests itself as reduced expression of insulin, insulin receptor, insulin-like growth factor 1 (IGF-1) and IGF1 receptor genes and insulin and IGF1 receptors, too. Elevated insulin degrading enzyme (IDE) gene expression in moderate AD and reduced IDE gene expression in progressed AD were found as well. Brain insulin resistance leads to a dysregulation of Aβ, increases the

possibility of tau hyperphosphorylation. Impaired insulin pathway results in a decrease in the number of glucose transporters, which leads to impaired energy metabolism, oxidative stress, mitochondrial dysfunction and pro-inflammatory cytokine activation.

The STZ model of AD

Regrettably, no new undoubtedly effective cognitive enhancer has been found in the last 20 years, despite the intense efforts. Research in the field of pharmacological therapies has been focused on the amyloid cascade hypothesis and relied on transgenic mouse models with β–amyloid overproduction. However, treatments based on beta amyloid antibodies have repeatedly failed during clinical trials or their effectiveness is questioned. Due to the series of failures, animal models of sporadic AD that do not involve genetic modification have regained attention in research. One of these alternative approaches is the intracerebroventricularly (icv.) injected streptozotocin (STZ) model.

The construct validity of the icv. STZ model is based on the induced insulin resistant brain state. According to the literature, STZ produces many symptoms of AD such as cognitive deficiency and increased phospho-tau at 1 month post-injection, elevated β-amyloid level at 3 months, appearance of plaques-like formations at 6 months. It appears to be a more adequate model than single transgenic mice and has the additional advantage of being applicable to rats, too. Nevertheless, it also has limitations (lack of NFTs, strain and sex differences), and its utility should be treated with caution until it is clear how the drug candidates found in this model perform in the clinic.

The rodent test battery system

Our research team established a test system for rodents, where the same animals are taught for several cognitive tasks and then

maintain their performance in regular training sessions. The cognitive tasks represent different cognitive domains , such as five-choice serial reaction time task (5-CSRTT) for attention, a cooperation task for social cognition, Morris water maze paradigm for spatial memory, "pot-jumping" exercise for procedural memory, pairwise discrimination for visual memory. We consider these learnt, "knowledgeable" animals a better model of the human population than naïve or freshly taught animals. Next, a certain impairment method can be applied in this population, and the impairing effect on the acquired cognitive functions can be simultaneously detected. Finally, efficacy of a putative cognitive enhancer treatment on the defective functions can then be studied in a "clinical trial-like", vehicle controlled, double blind, randomized experimental design.

The aims of the doctoral work

The objective of the doctoral work was to integrate the icv. STZ model as a particular impairment method to our test system. We implemented the model in several steps (see also the objectives). Since we use Long-Evans animals, while the literature mainly uses naïve albino rats, the first step was trying to reproduce the cognitive and biochemical changes described in Wistar rats in the literature in naive Long-Evans rats as well. The second step was the investigation of the icv. STZ treatment on young experienced Long-Evans rats to examine whether STZ has the same effect on experienced animals as on naïve rats. The third (final aim) was to study the effects of icv. STZ treatment in old experienced Long-Evans rats since theoretically, old experienced animals are translationally the most relevant population for the experimental investigation of AD. Patients with AD are typically elderly people and have complex knowledge due to their age.

2. Objectives

Our aim was to answer the following questions:

1. Does STZ cause the same behavioural and biochemical symptoms in Long-Evans rats as in Wistar rats?
2. Does STZ have the same effect on experienced animals as on naïve rats?
    a. Is there a difference in the sensitivity of cognitive functions to STZ treatment?
    b. What is the time course of cognitive deterioration after STZ administration?
3. What are the effects of STZ on old experienced animals?
    a. Is there a difference in the sensitivity of cognitive functions to STZ treatment?
    b. What is the time course of cognitive deterioration after STZ administration?

3. Methods

Studies in young unexperienced animals

During our first experiment (EXP1), a dose of 2x1.5 mg/kg STZ was used, based on the literature, and then, due to its ineffectiveness, a higher dose (3x1.5 mg/kg) was applied in subsequent study (EXP2). Eight-nine-week-old male Long-Evans rats were used in these studies; 18 subjects in experiment 1 (EXP1), and 24 in experiment 2 (EXP2). After STZ treatment, the animals' recognition memory (novel object recognition test-NOR), attention (5-Choice Reaction Time Task-5CSRTT), fear memory (passive avoidance learning test-PAL), and spatial memory (Morris water-maze test-MWM) were tested. At the end of the behavioural measurements the animals were sacrificed, their hippocampi were dissected for the western blot measurements. Phospho-tau and β-amyloid were chosen as disease markers, as they are the main pathological

biochemical hallmarks of the disease. The experiments lasted for 15 (EXP1) or 14 weeks (EXP2).

Studies in young experienced animals

Based on the previous experiments, we continued the examination of the effects of icv. STZ on young experienced animals with the dose of 3x1.5 mg/kg. Twenty-four 10-month-old male Long-Evans rats were used in this study. The animals were regularly trained in several learning paradigms for 8 months: 5-CSRTT for attention, a cooperation task for social cognition, MWM paradigm for spatial memory, "pot-jumping" exercise for procedural memory. After icv. STZ administration, the animals were re-tested in the learnt paradigms to see the – possibly time dependent - effect of the STZ treatment. Additional tasks, such as NOR for recognition memory, PAL and fear conditioning (FC) for fear memory were also introduced. Besides, spontaneous motor activity in open-field (OF) and elevated plus maze (EPM) performance for measuring anxiety were also examined. At the end of the behavioural measurements the animals were sacrificed and their hippocampi were harvested for the western blot measurements. The experiment lasted for 13 weeks.

Studies in old experienced animals

Examination of the effects of icv. STZ on aged experienced animals was also performed with the dose of 3x1.5 mg/kg. Twenty-nine 23-month-old male Long-Evans rats were used in this study. The animals had a long learning history in several paradigms for 21 months (the same tests as in the previous study). After the icv. injection surgeries, the animals were re-tested in the learnt paradigms to see the – possibly time-dependent - effect of the STZ treatment and additional tasks were also introduced (the same paradigms as in the young experienced group). To examine the ability to acquire new knowledge, pairwise visual discrimination learning was tested

for 9 days. At the end of the behavioural measurements the animals were sacrificed, their hippocampi were harvested for western blot measurements. The experiment lasted for 15 weeks.

Intracerebroventricular streptozotocin treatment

During EXP1 (studies in young unexperienced animals), 3 mg/kg icv. STZ divided into two 1.5 mg/kg doses were given bilaterally at day 1 and day 3. During EXP2 and the studies in young and old experienced animals, 4.5 mg/kg STZ split into three equal doses (1.5 mg/kg) was administered on days 1, 3, and 5. A volume of 2μL/ventricle was injected to the left and the right ventricle for a rat of 500 g. The dose was adjusted to the body mass of the animal by changing the injection volume. STZ was dissolved in 0.05 M citrate buffer pH 4.5.

Novel object recognition – recognition memory

The assay itself consisted of two trials, an acquisition trial and a retention trial. In the acquisition trial, the rats had 3 minutes to explore two identical objects in the experimental chamber. After a delay of 60 minutes, in the retention trial one of the objects was exchanged to a novel one and the animals had again 3 minutes to explore them. The measured variable was the animals' discrimination between the familiar and unfamiliar objects.

Passive avoidance learning – fear memory

A step through passive avoidance paradigm was applied. The apparatus consisted of a light and a dark chamber separated by a guillotine door. During the acquisition trial, the rat was placed into the light chamber from which it could cross into the dark chamber. Having done so, it received a mild foot-shock. Twenty-four hours later this procedure was repeated with the

exception that foot-shock was not delivered. The measured variables were the entry latencies into the dark compartment in the acquisition and the retention trials.

Morris water-maze – spatial memory

During the task, the animals needed to find a submerged hidden platform in a large pool filled with water using extra-maze cues for navigation. The rats escape latency was recorded during the task. In the case of the young unexperienced animals, after acquiring the task, a probe trial was performed when the hidden platform was removed from the pool, and memory trace was measured by the time the rats spent in the quadrant where the platform had been located during the acquisition trials. In the case of the trained groups, after acquiring the task the animals participated in regular maintenance sessions when the platform location was changed at each occasion.

5-choice serial reaction time task - attention

In this task, rats had to nose-poke into a hole out of five in which a light stimulus was turned on for 1 second. The animal made a correct response if it nose-poked into this hole during the stimulus presentation or within 5 s afterwards. Correct responses were rewarded with a pellet delivered into a food dispenser. Rats were trained for the task in stages with gradually decreased stimulus duration. The outcome variables were the percentages of correct responses, omissions (when the rat did not make any nose-poke in response to the light stimulus), premature responses (nose-poke into any of the holes during the inter-trial interval) and the accuracy $\left(\frac{total\ correct\ asnwers}{total\ correct\ answers + total\ incorrect\ answers} x100\right)$.

Pot jump test – procedural memory

In the MWM tank 12 flower pots were placed upside down forming a circle. Distance between the adjacent pots gradually

increased from 18 to 46 cm in anticlockwise direction. The tank was filled with water up to 5 cm to restrain rats climbing off the pots. During a session, animals were placed onto the start pot which was within the shortest distance from the next pot. For 3 min they could freely move on the pots and their behaviour was observed. Outcome variables were the longest interpot distance jumped over and the number of passes.

Cooperation – social memory

Social memory was measured in a cooperation task. The opposite walls of the chamber were equipped with one nose-poke module, one lever press module and one magazine for each. During the task, the animals worked in pairs but were separated from each other by a separating fence. When both nose-poke modules became illuminated one of the animals had to keep on nose-poking into its module for 3 s duration, which response activated the lever at the opposite side. The other animal then had to push the lever, as a result of which they both received a reward pellet and a new trial started.

Fear conditioning – fear memory

The experiment consisted of one acquisition and two retention trials (24 h and 1 month later). During the acquisition trial, the rats received 5 mild foot-shocks (0.6 mA, 1 s) as unconditional stimulus. The shocks were preceded by a combination of continuous sound and flickering light stimuli for 10 s, in the last second overlapping the unconditional stimulus. During retention trials, the animals received the same conditional stimuli, in absence of the foot shock. The main outcome variable was the animals' freezing time.

Elevated plus-maze - anxiety

The apparatus consisted of a plus-shaped platform with two open and two closed arms. The entire maze was 50 cm elevated

from the floor. The animals were placed in the middle of the platform, facing one of the open arms and had 300 s to explore the maze. The time spent in the open arms and the number of entries to the arms were measured.

Open field test – spontaneous motor activity

In this test rats were placed in a 48cm x 48cm x 40 cm (width x length x height) box equipped with an infrared beam net where the horizontal and vertical movements of the animals were recorded for 30 min. Analyzed variables were the ambulation time, local movement time and immobility time.

Pairwise visual discrimination

Subjects were trained to discriminate between two images presented randomly in the left or right window of a touchscreen apparatus. Touching one of the images resulted in a food pellet reward (correct response) while touching the other evoked timeout punishment (incorrect response). Number of completed trials, correct and incorrect responses were registered.

Western blot

Membranes were incubated with primary antibodies against phospho-tau (p-tau), tau and β-amyloid overnight at 4°C, followed by 2 h incubation at room temperature with anti-mouse HRP-linked secondary antibody. Phospho-Tau protein expression was normalized to the corresponding total protein. β -actin was used to control for sample loading and protein transfer and to normalize the content of the β-amyloid.

4. Results

In young unexperienced animals 2x1.5 mg/kg icv. STZ treatment did not result in significant difference between the control and STZ-treated groups, either in the behavioural assays or in the biochemical markers β-amyloid and phospho-tau/tau ratio (EXP 1).

In a subsequent study (EXP2) performed with increased dose of STZ (3x 1.5 mg/kg icv.) we found more pronounced effects in the NOR, 5-CSRTT, β-amyloid, and phospho-tau assays compared to EXP1. However, we observed no difference in the MWM and PAL tests.

In young experienced rats, STZ-treatment impaired recognition memory, spatial memory and attention. However, the impairment in the attention test was transient, as it passed by the end of the experiment and a similar trend was observed in the MWM as well. These findings indicate that the negative impact of the STZ treatment could be mitigated to some extent by the knowledge that was acquired beforehand. Impaired procedural memory was also found in STZ treated rats. On the other hand, there was no significant difference between the control and STZ-treated groups in the PAL and FC tests, and in the cooperation paradigm. STZ treatment increased novelty-induced exploratory activity in the open-field, but caused no significant difference in the anxiety levels of animals in the EPM. STZ differentially affected β-amyloid and phospho-tau levels: in the former no change could be observed while in the latter a non-significant, moderate increase was detected.

In the study with old experienced rats, 3x1.5 mg/kg STZ proved to be toxic to the old animals, as we lost four drug-treated rats during the post-treatment period. The treatment impaired

recognition and spatial memory whereas attention was not affected. The latter finding suggests that the knowledge accumulated over the years became resistant to the impairing intervention. Procedural memory of the rats was also not influenced by the treatment, possibly due to a floor effect since the old animals had already moved short distances in the pot-jumping test even before the study. Social memory could not be evaluated due to mortality and thus disintegration of pairs. Fear memory was not affected in the PAL test, but a marginally significant difference was found in the FC test. In the latter, STZ treated animals spent twice as much freezing as the controls during the retention trials. This apparent contradiction can be resolved if we assume that the intensity of freezing reflects not the strength of the memory trace but rather an increased level of anxiety related to the previously experienced shock. In the pairwise visual discrimination task both groups demonstrated similar learning efficiency in terms of the percentage of correct responses. However, rats treated with STZ initiated and completed a significantly higher number of trials compared to the controls. These results suggest that the rats' ability to acquire new knowledge was not disrupted by the treatment. A peculiar and notable finding in the STZ-treated group was the increased percentage of premature responses in the 5-CSRTT. STZ treatment increased novelty-induced exploration in the open-field test. Furthermore, STZ treated rats showed signs of decreased anxiety in the EPM test. β-amyloid and phospho-tau levels were affected differently, in the former no change could be detected while in the latter a significant, albeit moderate increase was observed. We assumed that the lack of an elevated amyloid level in the old animals could be due to a possible ceiling effect.

Table 1. The summary of the results obtained (x: not tested)

Test	**Young unexperienced rats**	**Young experienced rats**	**Old experienced rats**
Recognition memory	Decreased	Decreased	Decreased
Spatial memory	Unchanged	Decreased	Decreased
Procedural memory	x	Decreased	Unchanged
Attention	Decreased	Decreased (transient effect)	Unchanged
Fear memory	Unchanged	Unchanged	Unchanged
Visual discrimination	x	x	Unchanged
Social memory	x	Unchanged	x
Anxiety	x	Unchanged	Decreased
Basic activity	x	Increased	Increased
Impulsivity / motivation	x	Not observed	Increased
Phospho-tau	Increased	Increased	Increased
β -amyloid	Increased	Unchanged	Unchanged

Age dependence of β-amyloid level

In a separate measurement we re-assayed the tissue protein levels of β–amyloid in the control rats of all the three studies.

We found an age-dependent increase in β-amyloid level with significant differences between the three age groups (Fig. 1).

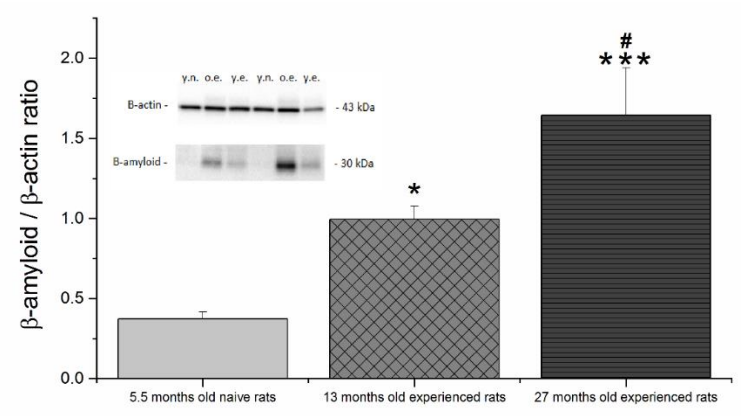

Figure 1. Comparison of tissue protein levels of β-amyloid in 5.5-month-old (young naïve, y.n.), 13-month-old (young experienced, y.e.) and 27-month-old (old experienced, o.e.) rats measured by western blot.

5. Conclusion

The main conclusion of the study with young unexperienced rats was that Long-Evans rats are likely less sensitive to STZ treatment than albino rats, but 3 × 1.5 mg/kg STZ was sufficient to induce subtle behavioral and biochemical changes. These findings suggest that icv. STZ treatment may develop a different pathology in Wistar vs Long-Evans strain.

Using experienced rats in the second study allowed longitudinal following the effect of STZ. The examined cognitive domains showed different sensitivity to STZ and the impairing effects of

the compound faded away by time in case of previously learnt responses.

Our third study revealed that STZ treatment differently affected the young and old experienced Long-Evans rats. Furthermore, its lack of effect on β–amyloid level – possibly due to the age-dependent plateauing of the protein level found in the auxiliary study – suggests that its impairing cognitive effects may not be mediated by increasing β-amyloid level. However, the most interesting finding of this study was the marked emotional effects of STZ, interpreted – as a working hypothesis –as impulsivity. The observed behavioural and molecular activity profile (impulsivity and lack of elevated β-amyloid) hints at a possible frontotemporal dementia (FTD) connection.

The obtained results and our literature survey suggest that icv. STZ is not a superior model of AD. It has certain promising features but also has several flaws. A deficiency of the literature is that most of the studies used young, typically 3-month-old animals and there are only a few studies where aged rats were used. This is problematic since AD is a disease of old age. Our results also pointed out at the age dependence of the treatment. Important to highlight that the one-month long studies are not adequate for modeling a slowly developing, gradually progressing chronic neurodegenerative process. It is of concern, that in most of the articles just one or two behavioral tests (typically MWM and/or PAL) were used, which does not give a comprehensive picture of cognitive deterioration.

Translation-wise, old animals with learning experience would be the most adequate subjects for modeling AD. To the best of our knowledge, this was the first study that examined the effects of icv. STZ in trained aged Long-Evans rats. Using this particular population may offer a solution to some of the above-mentioned problems with the model, but even our approach needs further elaboration. It would be necessary *i)* to expand the

tested cognitive domains; *ii)* to check the development of insulin resistance; *iii)* to investigate the relationship between the STZ model and FTD; *iv)* to examine the effect of STZ in experienced old female rats; and *v)* in experienced old Wistar rats. Last, but not least, it would be also necessary to repeat the experiments with another group of old experienced Long-Evans rats to see whether it is possible to reproduce the results. These proposed studies represent the most appropriate way to strengthen and specify the translational validity of the icv. STZ model.

6. Bibliography of the candidate's publications

Own publications involved in the current thesis

1. Gáspár A, Hutka B, Ernyey AJ, Tajti BT, Varga BT, Zádori ZS, Gyertyán I. Intracerebroventricularly Injected Streptozotocin Exerts Subtle Effects on the Cognitive Performance of Long-Evans Rats. Front Pharmacol. 2021;12:1–11.

2. Gáspár A, Hutka B, Ernyey AJ, Tajti BT, Varga BT, Zádori ZS, Gyertyán I. Performance of the intracerebroventricularly injected streptozotocin Alzheimer's disease model in a translationally relevant, aged and experienced rat population. Sci Rep. 2022;1–13.

Own publications not involved in the current thesis

1. Varga BT, Gáspár A, Ernyey AJ, Hutka B, Tajti BT, Zádori ZS, Gyertyán I. Introduction of a pharmacological neurovascular uncoupling model in rats based on results of mice. Phys Int. 2022;109(3), 405-418.

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