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

PhD thesis

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List of abbreviations

5-CSRTT	five-choice serial reaction time task
AD	Alzheimer's disease
AICD	APP intracellular domain
AKT	protein kinase B
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APOEε4	ε4 allele of apolipoprotein E
APP	amyloid precursor protein
ATP	adenosine triphosphate
Aβ	amyloid beta
C99	APP C-terminal fragment 99
DI	discrimination index
DNS	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
EPM	elevated plus maze
EXP	experiment
FC	fear conditioning
FDA	Food and Drug Administration
FTD	frontotemporal dementia
GABA	gamma aminobutyric acid
GLUT2	glucose transporter 2
GSK3 α/β	glycogen synthase kinase 3α/β
icv.	intracerebroventricular
IDE	insulin degrades enzyme
IGF	insulin-like growth factor
IR	insulin receptor
IRS	insulin receptor substrate
LTD	long-term depression

LTP	long-term potentiation
MAPT	microtubule-associated protein tau
MWM	Morris water-maze
NAD+	nicotinamide adenine dinucleotide
NFTs	neurofibrillary tangles
NMDA	N-methyl-D-aspartate
NOR	novel object recognition
NW	north west
o.e.	old experienced rats
OF	open-field
O-GlcNAc	O-N-acetylglycosamine
PAL	passive avoidance learning task
PD	pairwise visual discrimination
PHF13	paired helical filaments 13
PI3-K	phosphatidylinositide 3-kinase
pTau	phospho-tau
s	secundum
sAPP	soluble amyloid precursor protein
SE	south east
STZ	streptozotocin
WB	western blot
y.e.	young experienced rats
y.n.	young naïve rats

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1. Introduction

1.1. 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 (1,2). Unfortunately, AD is relentlessly progressive and currently there is no known medical treatment that would cure the disease. Research for treatment options is also hindered by the fact that the exact cause of its development is still not known. The bitter feature of the disease is that, due to cognitive decline, it not only ruins the lives of the patients, but also seriously impairs the quality of life of the family members who take care of them. The growing number of patients (due to lack of adequate medication) poses serious economic and healthcare problems in the near future. In order to improve the living conditions of patients (and their relatives), it is particularly important to develop medicines that would achieve a significant improvement in the quality of life (1–3). Better understanding of the disease, development of new approaches, new therapies, surgical procedures, and experimental models are all essential for the development of an effective drug or non-drug treatment (4). Although we do not know the exact cause of AD, we are aware of several risk factors that can lead to the development of this malady. Such risk factors are the family history of dementia, head trauma, genetic factors (dominantly inherited mutations in amyloid precursor protein (APP), presenilin 1 and presenilin 2, the ε4 allele of apolipoprotein E (APOEε4)), high blood pressure, low education level and environmental factors, but the most characteristic risk factor is advanced age. The main histological hallmarks of the disease are senile plaques and neurofibrillary tangles (NFTs) (1–3). The most common cognitive symptoms are memory impairment, executive dysfunction, aphasia, apraxia, and agnosia. Progressive cell death of neurons leads to cerebral cortex shrinkage, enlarged ventricles (Fig. 1) and lesions in the amygdala, subiculum, hippocampus and entorhinal cortex (1,3).

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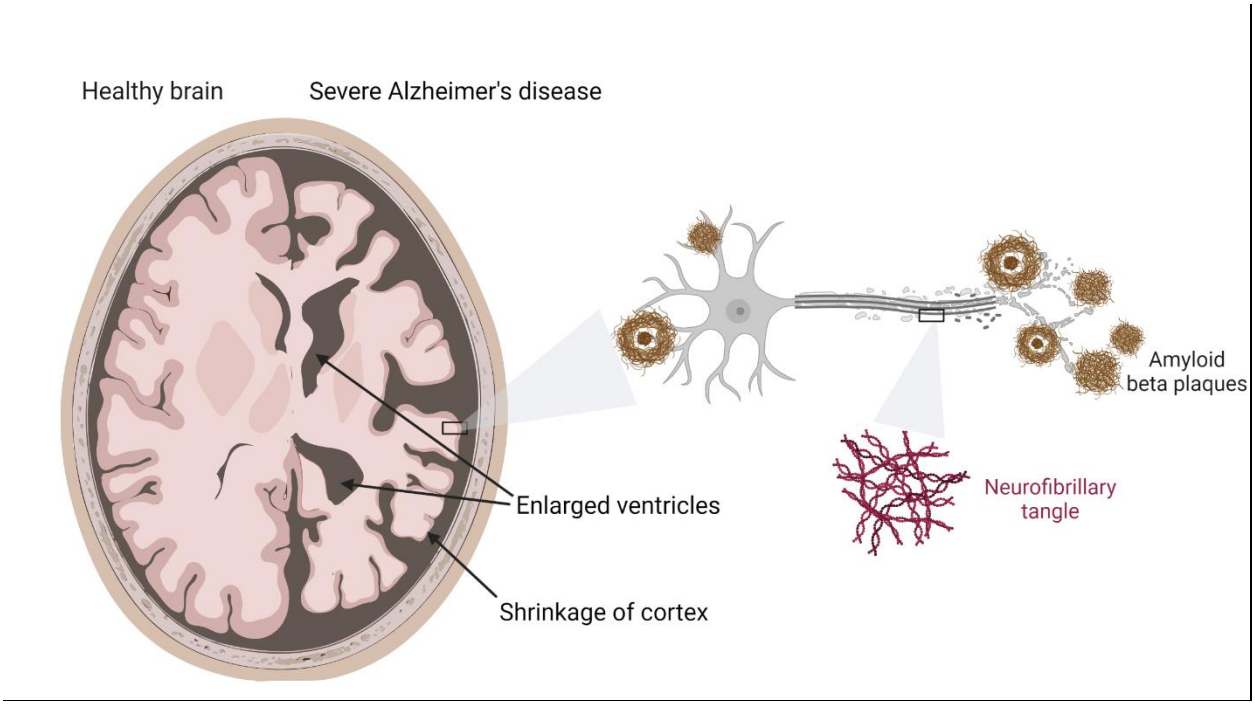

Figure 1. Brain changes in Alzheimer's disease (created with BioRender.com).

Clinically, we could distinguish two types of the disease. The familial form, which accounts for 4-8% of all AD cases, and the sporadic form, which is responsible for the majority of cases (5). Many hypotheses have been made about the pathomechanism of the illness, although the exact cause is still unknown. Without claiming to be complete, I would like to present a few recognized ones.

1.2. Hypotheses of Alzheimer's Disease

1.2.1. Cholinergic hypothesis

One of the widely recognized hypotheses, which still forms the basis of drug treatment today is the cholinergic hypothesis. It suggests that the cognitive impairment is a consequence of the degeneration of the ascending cholinergic pathway. Acetylcholine is a neurotransmitter, participate in attention, learning, memory, stress response, wakefulness, sleep, and sensory information (6). The loss of cholinergic neurons and the cholinergic system failure during AD is proven (reduced choline acetyltransferase activity, acetylcholine synthesis, choline uptake and acetylcholine release) (5), which leads to the alteration of cognitive functions and memory loss. Based on these findings, cholinesterase inhibitors are widely used as a treatment method. Unfortunately, the

efficacy of the cholinesterase inhibitors is low and transient. Due to lack of lasting improvement or at least halting the disease progress, it is a symptomatic treatment rather than a cure (5–7).

1.2.2. Amyloid cascade hypothesis

The amyloid cascade hypothesis is one of the most accepted one and has been in the focus of drug research for the last decades (6). The hypothesis suggests that amyloid-β (Aβ) generation and aggregation is the triggering factor of AD. Its theoretical basis is the abnormal accumulation of the β-amyloid peptide, which assemble into soluble amyloid-β oligomers. These soluble oligomers can trigger processes (amyloid cascade) leading to the death of neurons and can aggregate into insoluble fibrils, which tend to deposit in plaques extracellularly. The β-amyloid peptides are produced by the incorrect cleavage of the transmembrane amyloid precursor protein (APP) by the β and the γ secretase enzymes. Normally, APP participates in synapse formation. During AD, β secretase cleaves APP to an extracellular N-terminal and an intracellular C-terminal fragment which binds to the cell membrane. The C-terminal fragment is then cleaved by the γ secretase resulting in the release of the Aβ protein (Fig. 2). Aβ deposition and diffuse plaques lead to local microglial activation, cytokine release, intracellular calcium (Ca^{2+}) dysregulation, reactive

astrocytosis, development of an inflammatory response, synapsis and neuron loss (1,5,8) and also develop cerebral amyloid angiopathy in the walls of cerebral blood vessel (3,9–11).

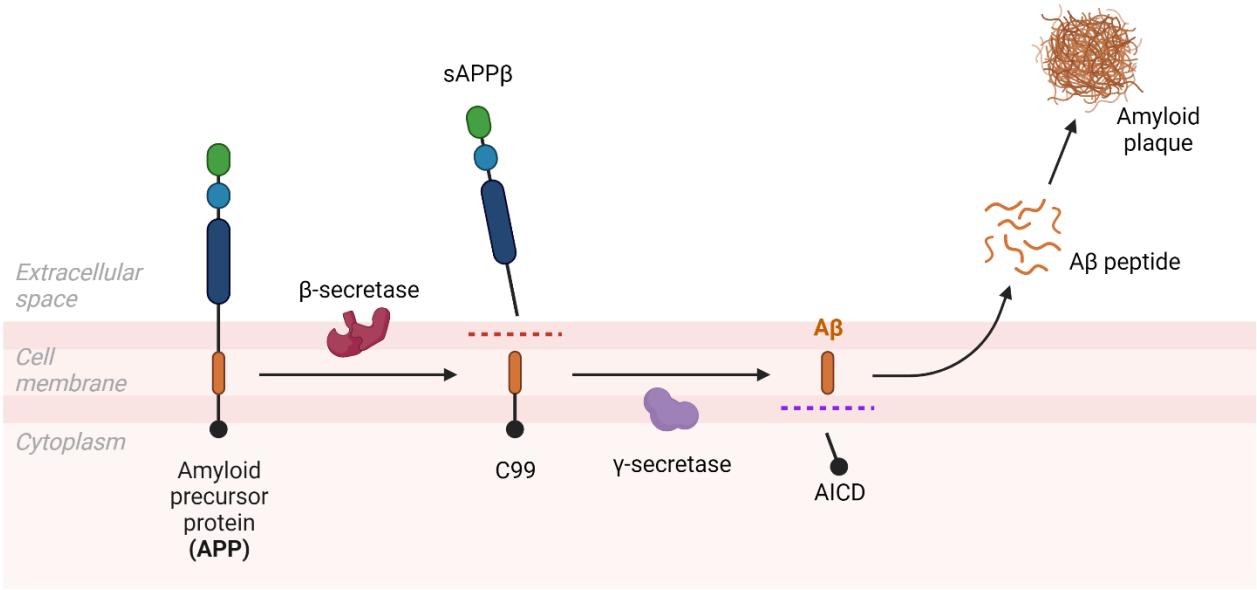

Figure 2. The amyloidogenic APP processing pathway (APP: amyloid precursor protein; sAPP-α/*β:* soluble amyloid precursor protein-α/*β;* A*β:* amyloid beta; C99: APP C-terminal fragment 99; AICD: APP intracellular domain (created with BioRender.com).

1.2.3. Tau hypothesis

Another accepted hypothesis is the tau hypothesis. Tau proteins are microtubule-associated proteins that participate in the stabilization of microtubules. AD is classified as a tauopathy because tau is abnormally phosphorylated during the course of the disease (3). Hyperphosphorylation leads to the detachment of tau from the microtubule and formation of neurofibrillary tangles and neuronal degeneration (Fig. 3). The hyperphosphorylated tau is resistant against proteolysis (12). Intraneural accumulation of fibrillar tau leads to cytoskeletal network and axonal transport disruption. Overall, the loss of normal function of tau results neuronal dysfunction and cell death (3,11).

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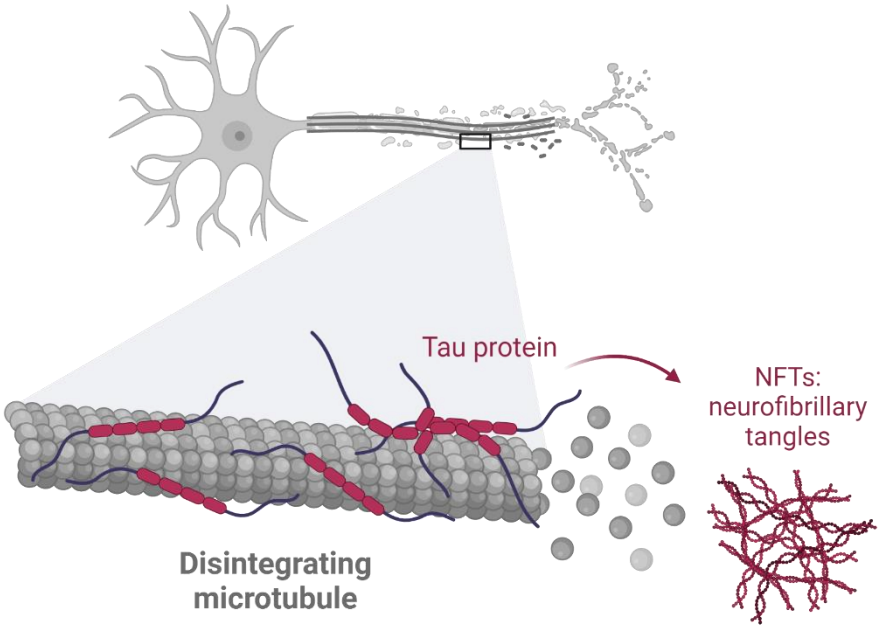

Figure 3. Tau hyperphosphorylation during AD (NFTs: neurofibrillary tangles) (created with BioRender.com).

1.2.4. Type 3 diabetes hypothesis

In addition to the above-mentioned and currently most accepted hypotheses, there are many other, more or less widely accepted theories. One hypothesized cause is the insulin resistant brain state, present during AD, why it is also called – although misleadingly- Type 3 diabetes (13,14). Insulin and insulin-like growth factor (IGF) signaling pathway play a crucial role in cognitive functions and regulate extensive neuronal functions through ligand-receptor binding activation of receptor tyrosine kinases. Interactions between the insulin receptor substrate molecules and the phosphorylated receptors mediate several neuronal functions, like growth, survival, metabolism, and plasticity. Insulin regulates the expression and levels of gamma aminobutyric acid (GABA), N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors thereby affects long-term potentiation (LTP) and long-term depression (LTD) (15–17). Insulin has an important role in the utilization and metabolism of glucose by neurons and in the synthesis of adenosine triphosphate (ATP), too. In a heathy brain, insulin receptor signaling activates phosphatidylinositide 3-kinase (PI3-K), which activates protein kinase B (AKT). Protein kinase B/AKT is participating in the regulation of glucose metabolism, generation of ATP and inactivation of glycogen synthase kinase 3α/β (GSK3 α/β) also (18). GSK3 is a serine-threonine

kinase and has a role in cellular proliferation, migration, glucose regulation, apoptosis, and regulates the production of Aß peptides (through the modulation of APP cleavage) and tau phosphorylation (able to phosphorylate tau).

Impaired glucose metabolism may also contribute to tau hyperphosphorylation via the impaired hexosamine biosynthetic pathway which normally leads to the generation of O-N-acetyl-glycosamine (O-GlcNAc). O-GlcNac is used for acylating proteins (called O-GlcNAcylation) as part of posttranslational protein modification. Both O-GlcNAcylation and phosphorylation use the serine or threonine sites of a protein. The functional role of tau O-GlcNAcylation is not exactly known but the results show that it is protective against tau aggregation, as it competes for the same amino acid residues, thereby reduces the possibility of tau hyperphosphorylation (16,18,19). Human postmortem studies show signs of insulin resistance during AD (13,15,20–22). It manifests itself as reduced expression of insulin and insulin-like growth factor 1 (IGF-1) genes, insulin and IGF1 receptor genes and insulin and IGF1 receptors, too (13,20,21). Elevated insulin degrading enzyme (IDE) gene expression in moderate AD and reduced IDE gene expression in progressed AD were found as well (22). Brain insulin resistance 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. Mitochondrial dysfunction generate reactive oxygen species, which leads to deoxyribonucleic acid (DNA) modification and cellular organ damage. During impaired insulin/IGF signaling (Fig. 4), the (PI3K)-AKT signaling activity is inactivated which leads to reduced phosphorylation and inappropriate overactivation of GSK3α and GSK3β. While the activated GSK3α leads to a dysregulation of Aβ by modulating the APP cleavage, the activated GSK3β leads to tau hyperphosphorylation. The impaired insulin signaling causes a decrease in glucose/energy metabolism, therefore reduction of ATP synthesis; furthermore, O-GlcNAcylation is also diminished, which increases the possibility of tau hyperphosphorylation. Overall, these pathological cellular processes results in cell death (8,15,16,18).

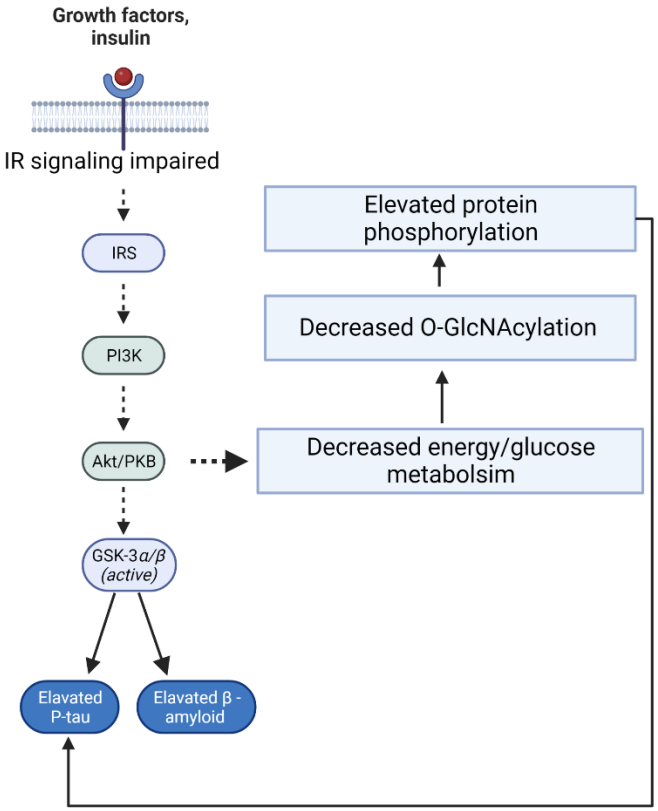

Figure 4. The insulin receptor signaling impairment (IR: insulin receptor; IRS: insulin receptor substrate; PI3K: phosphatidylinositide 3-kinases; Akt/PKB: protein kinase B; GSK3α/β: glycogen synthase kinase 3α/β). During insulin signal impairment, IRS and PI3-K pathway is impaired. This dysfunction leads to reduced Akt/PKB activity, which results in decreased energy metabolism, decreased O-GlcNAcylation and reduced phosphorylation of GSK3α/β. Over activation of GSK3α/β results in tau phosphorylation and β -amyloid production. Dotted arrows illustrate reduction/inhibition, while solid arrows illustrate stimulation of the processes (created with BioRender.com).

1.3. Lack of cognitive enhancers

Regrettably, no new undoubtedly *effective* cognitive enhancer has been found in the last 20 years (23–25), despite intense research. Since 2003, more than 200 therapeutic agents examined in clinical trials have been failed or abandoned (26). Currently 143 drug candidates are in the AD drug development pipeline, 31 agents in Phase 3, 82 agents in Phase 2, and 30 agents in Phase 1 (27). Three families of drugs are used to treat AD (date of approval in parentheses): cholinesterase inhibitors, including donepezil (1996), rivastigmine (1998), and galantamine (2001), the NMDA receptor antagonist memantine (2003, its combination with donepezil is also used to treat the

disease) and the Food and Drug Administration (FDA)-approved monoclonal beta amyloid antibodies, aducanumab (2021, approved for patients with mild cognitive impairment or mild AD) and lecanemab (2023, for the treatment of early AD) (23,25,27,28). The most frequently used drugs are the cholinesterase inhibitors, despite that they provide symptomatic treatment. The theoretical background of the effectiveness of memantine is that the overactivated NMDA receptors cause a pathological level of Ca^{2+} influx and signaling, which leads to increased glutamate release and consequent overactivation of glutamate receptors, thereby causing excitotoxicity and cell death (1). Aducanumab targets aggregated beta amyloid while lecanemab selectively binds to large, soluble Aβ protofibrils (23,25,27,28). *N.B.:* the FDA decision on aducanumab was very controversial, made on the contrary of the advisory panel opinion (29), and although lecanemab produced more promising results compared to the former, its effectiveness is also doubtful (30). In recent years, research in the field of pharmacological therapies has been focused on the amyloid cascade hypothesis. Treatments based on beta amyloid antibodies have repeatedly failed during clinical trials or their effectiveness is questioned (see above) (31). Unfortunately, it is typical of all licensed drugs that their effect does not reverse or prevent the development and progression of AD (32). Current drug developments mainly focus on therapies targeting tau protein, anti-inflammatory processes, oxidative stress and still Aβ cascade (27,32).

1.4. Inappropriate animal models

Development of animal models that are more relevant to AD is crucial for improving our understanding of the disease and for developing drugs more efficiently. The inadequate predictive value of animal models for human efficacy is one of the reasons for the serial failures of drug candidates. In the case of drugs developed for symptomatic treatment, the weak point was the use of models where efficacy against transmitter-specific agents (e.g. scopolamine, phencyclidine) was the desired outcome. Due to their nature, these are of limited relevance, their relationship with the given disease is often hypothetical, and therefore their predictive power is low (33–35). Disease modifying drugs that farthest progressed in the pipeline – but ultimately failed - targeted the β-amyloid cascade (36). They had been selected by transgenic mouse models of the familial form of the disease (37). The transgene in these mice produced a large amount of human β-amyloid, but this process much more mimicked an exogenous amyloid intoxication than the disease pathology itself, since tau pathology was lacking and even the observed cognitive changes showed no

correlation with the histological findings. A series of clinical trial failures (38) and the fact that several AD patients produced memory deficits without amyloid plaques (37,39), raised serious doubts not only about the validity of transgenic models, but also about the validity of the amyloid theory itself. These findings questioned whether amyloidosis is the underlying cause of the disease (25,37,39). Recognizing this, multiple transgenic mouse models have been developed to mimic AD pathology, such as 5xFAD mice (three mutations in the APP gene and two in PSEN1 gene) and 3xTg Mice (containing three mutations associated with familial Alzheimer's disease: APP, microtubule-associated protein tau (MAPT), and PSEN1) (35,40). Currently, there are more than 170 transgenic mouse AD models simulate Alzheimer's disease. (35). Transgenic rats represent a new, maybe more promising direction of research. For example, TgF344-AD rats, having mutant human APP and presenilin-1 transgenes show so far the most complete AD pathology: accumulation of β-amyloid, amyloid plaques, increased phospho-tau and NFTs, neuronal loss, activated microglia and age dependent deficits in learning and memory (41). However, proving the utility of these new transgenic lines is still ahead of us.

1.5. The streptozotocin (STZ) model of AD

Due to the series of failures mentioned above, 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 (42) (see above for its theoretical background). According to the literature, it produces many symptoms of AD such as cognitive deficiency and increased phospho-tau at 1 month, elevated β-amyloid level at 3 months, appearance of plaques-like formations at 6 months post-injection (43). It appears to be a more adequate model than single transgenic mice (44) and has the additional advantage of being applicable to rats, too. Although, it seems to be a more promising model than genetically modified animals, it has its limitations (lack of NFTs (43), strain (45–47) and sex differences (48,49)), too

and its utility should be treated with caution until it becomes clear how the drug candidates found in this model perform in the clinic.

1.5.1. The STZ compound

STZ (2-deoxy-2-(3-(methyl-3-nitrosoureido))-D-glucopyranose) is a glucosamine-nitrosourea compound (Fig. 5), delivered from the *Streptomyces achromogenes* bacteria and originally developed as an anticancer drug (used as a treatment against islet cell carcinoma of the pancreas) (16,50). Besides being a sporadic AD model, STZ is mainly used as a diabetes mellitus model (though at much higher doses), since it selectively destroys insulin secreting pancreatic β-cells. STZ can transported to the cells via glucose transporter 2 (GLUT2) due to its similarity to glucose (STZ has an exclusive selectivity to GLUT2 transporters). GLUT2 is mainly expressed by the pancreatic β cells, kidney, liver and neurons. The icv. injected STZ does not cause elevated blood glucose and insulin levels since GLUT2 is not expressed in the blood brain barrier (16) therefore, it is not released from the brain; furthermore, the icv. dose is much lower than that used to induce systemic diabetes. Based on the literature icv. STZ treatment reduces cerebral glucose utilization (13) and the levels of the expression of insulin and IGF-1 receptors (16). The exact mechanism of action is not completely known but STZ exerts its damaging effect by DNA alkylation which results in cell death. The DNA alkylation leads to poly(ADP-ribose) polymerase activation, depletion of nicotinamide adenine dinucleotide (NAD+) and ATP stores. The neurodegeneration may develop via the impaired PI3K-AKT signaling activity and the overactivation of GSK3α and GSK3β (50,51).

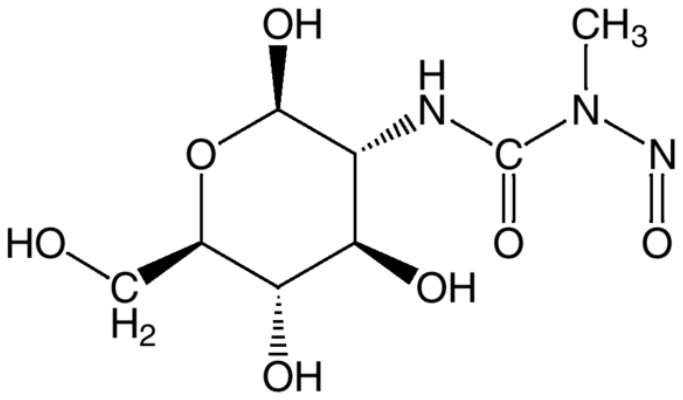

Figure 5. Structural formula of STZ.

1.5.2. The use of the icv. STZ model in the literature

Although the icv. STZ-induced brain pathology has been an increasingly used model of Alzheimer's disease, there is no clear agreement on the optimal dose (52,53), behavioural tests or rat strain (52).

The preferred subjects of the model are mainly albino rat strains (Wistar or Sprague-Dawley, rarely Lewis rats) (54) or mice (55) and there are only few studies from Long-Evans rat strains (54). The most common age of the animals during the treatment is 3-6 months and there are only a few articles in which older animals of 22-24 months were examined (56–59). In the sparse Long-Evans literature, 3-day-old Long-Evans pups (60–62) or 4-weeks-old young Long-Evans rats (22,63–66) rarely 10-week-old animals (67) were used.

The most common dosing of STZ is 3 mg/kg split into two 1.5 mg/kg doses injected with two days difference (10,58,68–74), but single, less than 1 mg/kg (22,61–66), 1 mg/kg (69,75–77), 1.5 mg/kg (78–80), 2 mg/kg (81), 3 mg/kg (82–89) and 6 mg/kg (90) doses are also applied. In one study, 2x 4.5 mg/kg was applied (67). The duration of observation after the injection also shows significant diversity. Most of the studies applied a one-month-long post-injection period (72,86,89,91,92) or 2-3 weeks intervals (68,74,78–80). Some studies used longer (3 months or more) follow-up periods (10,43,69,73,76,84,93,94). Two informative longitudinal studies (43,87) monitored changes and impairments for up to 9 months.

The most common cognitive tests in the literature are the passive avoidance learning task (PAL) (fear memory) and the Morris water-maze (MWM) (spatial learning and memory) tests. Their timing varies but impairments were shown already at 2-3 weeks post-injection both in the PAL (43,68,70,78,80,84,95,96) and in the MWM (58,69–71,74,75,84,92,97,98) tests. Learning deficit was also found at later measurement points (1 to 3 months) both in PAL (72,73,84,88,99) and in MWM (69,73,75,76,82,84,86,88,91,100,101). Impaired visual recognition memory in the novel object recognition (NOR) paradigm was observed at 3-8 weeks after STZ-treatment (72,81,89,99,102).

Results indicating insulin resistance are common findings in the STZ literature. Decreased insulin receptor gene expression (75), insulin receptor (100,103,104), IGF receptor (100) and IDE (69) levels were detected. In a very informative study, reduced level of IDE was found after 1, 3, 6, 9

months post treatment together with decreased insulin receptor level at 1, 3, 6 months (87). Lower levels of insulin receptor substrate (IRS) and IGF receptor were also reported in STZ-treated Long-Evans rats (13,66). However, unaltered IR expression was also found in Wistar (79,97) as well as in Long-Evans studies (66).

Increased phospho-tau /tau ratio was already observed from 2 weeks post-injection and was detected either by western-blot (69,75,76,81,86,87,89,91,92,99,105) or by immunostaining (73,99,102). Increase in β-amyloid level was observed at later timepoints, about 1.5 months on, by either enzyme-linked immunosorbent assay (ELISA) (84,86,93,99,102,105), western-blot (89,104,106) or immunostaining (43,69,76,104,106). Amyloid-plaque like deposits were observed first at 3 months after STZ treatment in the meningeal vessels detected either by congo red (76) or immunostaining (45). These plaque like deposits became more pronounced at 6 and 9 months (10) and also appeared in the brain parenchyma (43).

1.6. The rodent test battery system applied by our group

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 (34,46,107–109). The cognitive tasks represent different cognitive domains , such as five-choice serial reaction time task (5-CSRTT) for attention (110), a cooperation task for social cognition (111), Morris water maze paradigm for spatial memory (112), “pot-jumping” exercise for procedural memory (113), pairwise discrimination for visual memory (114). 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. As the system imposes heavy cognitive load on the subjects, Long-Evans rats were used for our experiments as this strain is well-known for its good learning capability (122–126). The animals are kept under restricted food access as studies have demonstrated that food restriction can promote better health and increase cognitive functioning (115–117) while slow down the aging process and reduce the mortality rate (118–121), compared to *ad lib* feeding. Furthermore, this regime makes the rats motivated to work in the food-rewarded tasks on each day.

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1.7 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 described above. 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 / objective 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.

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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?

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3. Methods

Methodical details (animals, description of the behavioural and biochemical assays, statistical analysis, etc.) can be found in the attached papers of Gáspár et al. (46) and Gáspár et al. (47). Nevertheless, study design will be shown at each study, and the applied cognitive paradigms are briefly introduced at the beginning of the appropriate paragraphs.

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4. Results

4.1. 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 (3x 1.5 mg/kg) was applied in another study (EXP2). Eight-nine weeks-old male Long-Evans rats were used in these studies; 18 rats in experiment 1 (EXP1), and 24 rats in experiment 2 (EXP2). After STZ treatment, the animals' recognition memory (NOR), attention (5-Choice Serial Reaction Time Task-5-CSRTT), fear memory (PAL), and spatial memory (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) (Fig. 6) (47).

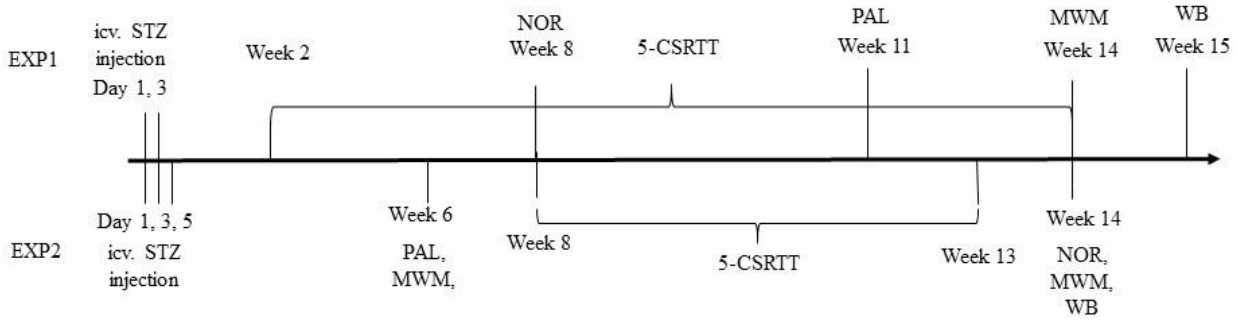

Figure 6. Timeline of the experiments in young unexperienced rats (EXP: experiment; icv: intracerebroventricular; STZ: streptozotocin; NOR: novel object recognition; 5-CSRTT: five choice serial reaction time task; PAL: passive avoidance learning; MWM: Morris water maze; WB: western blot) (47).

Novel object recognition (NOR)

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 80 minutes (EXP1) or 60 minutes (EXP2), in the retention trial one of the objects was changed to a novel one and the animals had 3 minutes again to explore them. The measured variable was the animals' discrimination between the familiar and unfamiliar objects (47).

In the first experiment (EXP1), STZ-treated animals explored less the unfamiliar new object than the control rats (Fig.7A) and their discrimination index (DI) value was also much lower (0.33 and 0.08 in the control and STZ group, respectively, Fig. 7B). Nevertheless, due to the low number of animals remained in the experiment (rats which explored the objects for less than 10 seconds or explored only one of the two objects in any of the trials were excluded from the experiment) the difference was not statistically significant (Fig. 7B). In the second experiment (EXP2), control animals spent significantly more time with examining the unfamiliar object (24.9 s) than the old one (13.9 s) whereas STZ-treated rats equally explored both (15.6 s and 15.8 s for new and old, respectively) (Fig. 7C). The DI values of the two groups (0.32 s and 0.05 s for control and STZ, respectively, Fig. 7D) were significantly different (47).

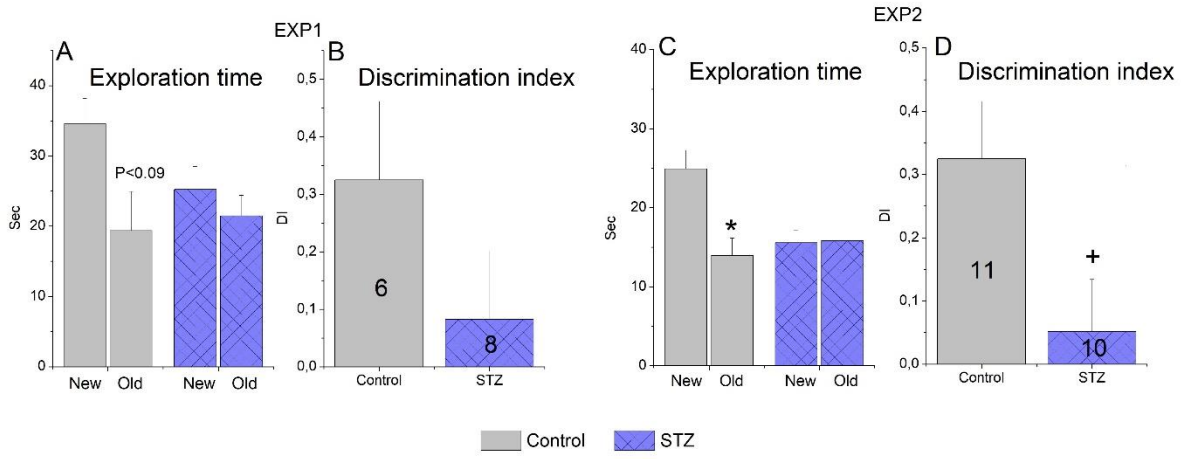

Figure 7. Novel Object Recognition performance of rats treated with icv. streptozotocin (STZ) or citrate buffer (control). Means ±SEM values are shown. Numbers inside the columns indicate the number of animals. (A) and (B) Results of experiment 1 (EXP1), when the animals received 2x1.5 mg/kg icv. STZ 8 weeks before the test. (C) and (D) Results of EXP2.* $p<0.05$ vs 'new' (paired t-test, $t(10)=3.53$); +< $p<0.05$ vs 'control' (unpaired t-test. $t(19)=2.21$) (47).

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Passive avoidance learning (PAL)

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. Animals which did not cross to the dark chamber at the acquisition trial were excluded from the experiment (47).

There was no significant difference between the learning performances of the groups either in acquisition or retention trials in any of the experiments (Fig. 8A, B) (47).

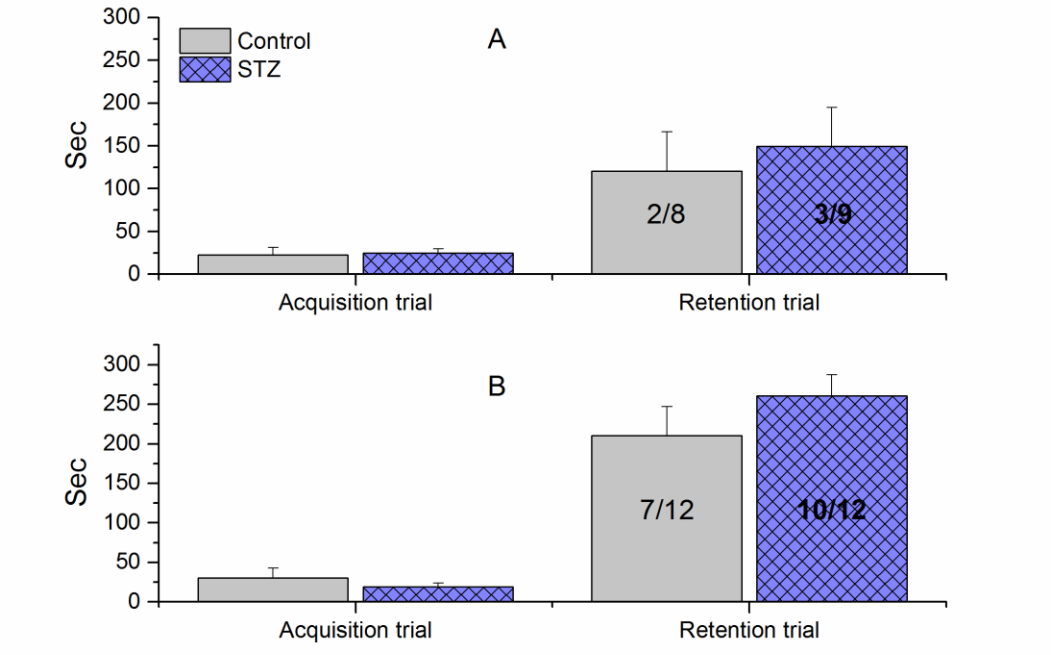

Figure 8. Passive Avoidance Learning results of rats treated with icv. streptozotocin (STZ) or citrate buffer (control). Columns show means ± SEM values of entry latencies, numbers inside the columns indicate the not entered/total number of animals. A: Results of experiment 1 (EXP1), B: Results of experiment 2 (EXP2) (47).

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Morris water-maze (MWM)

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. 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 (47).

In EXP1, control and treated animals similarly performed in the acquisition trials (days 1-2, Fig. 9A). All of the rats successfully learned the location of the hidden platform with similar decrease in their escape latency. The animals spent the same amount of time in the target quadrant during the probe trial. Furthermore, no significant difference was found between the groups during the re-acquisition trials, when the platform was relocated to a new position (Fig. 9A). In EXP2, again, no significant difference was detected between the performance of the control and STZ-treated groups in the three phases of the test. To examine the possible later development of cognitive impairment, an additional acquisition session and probe trial were carried out at week 14; nonetheless there was no significant difference between the groups (Fig. 9C) (47).

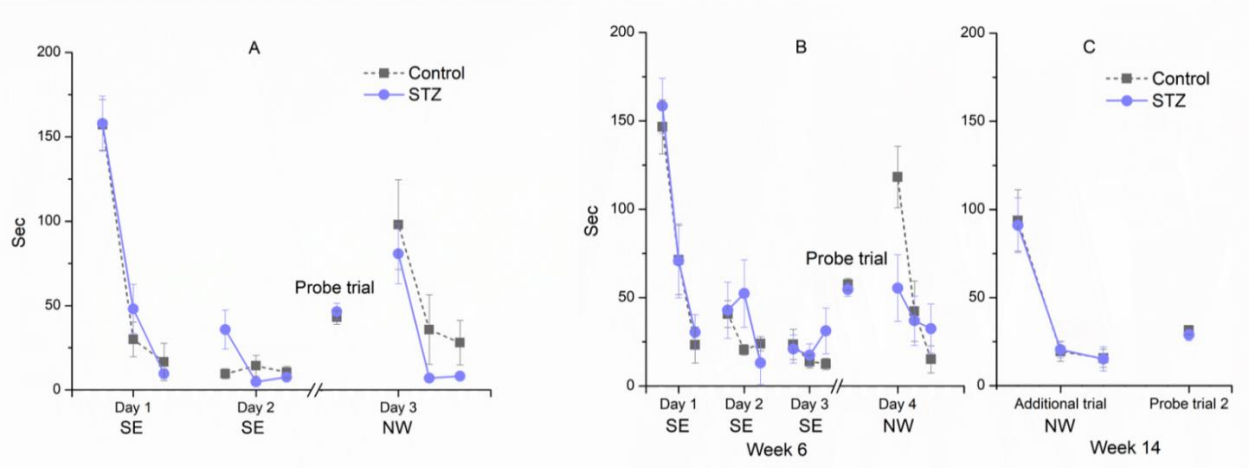

Figure 9. Learning performance in the Morris water-maze in EXP1 (A) and in EXP2 (B, C). Means ± SEM of escape latency values are shown except in the probe trial where the time spent in the target quadrant is depicted. SE and NW indicate the position of the escape platform (47).

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5-Choice serial reaction time task (5-CSRTT)

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. In the case of naïve rats, the outcome variables were the days needed to complete the final stage and the learning curve plotted as average learning stage in function of training days. The difference between learning curves was examined using the sigmoid curve fitting method (47).

In EXP1, STZ-treated and control animals showed intersecting and overlapping learning curves (inflection points of the fitted sigmoid regression curves were 24.7 and 25.8 days, respectively) and the days needed to reach the maximum learning stage were the same (43.8 and 43.8 days, respectively) (Fig. 10A). In EXP2, however, the control group learned significantly faster shown by the two days difference in the midpoint of the fitted sigmoid regression curves (11.4 and 13.4 days in the control and STZ-treated group, respectively, Fig. 10B), furthermore, the STZ-treated animals needed 3 days more to complete the task (21.2 days vs 18.0 days in the control group), though this difference was not significant (47).

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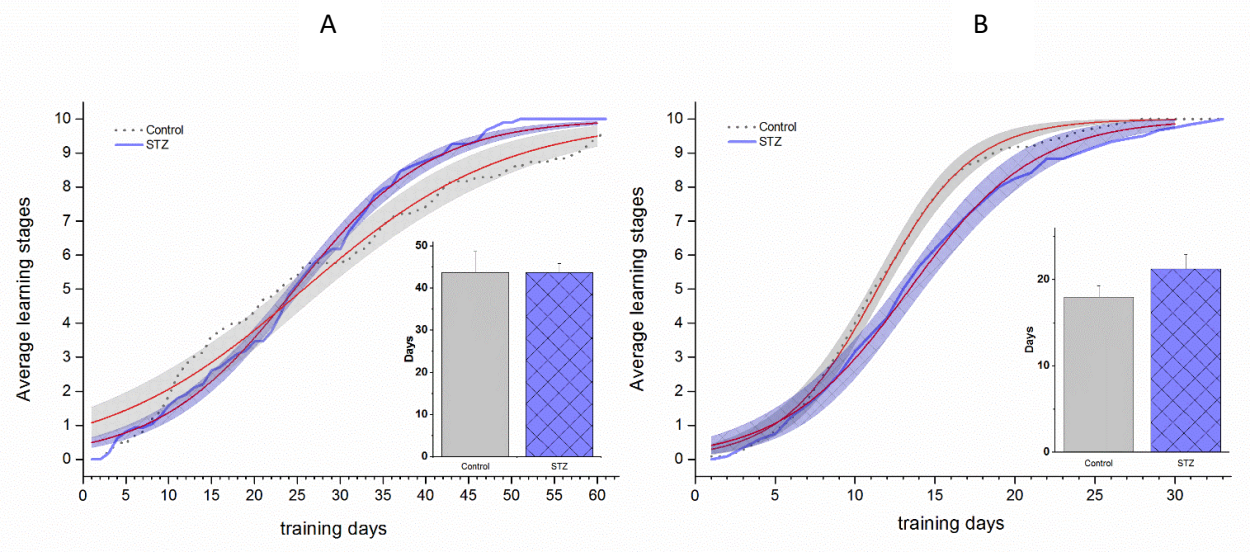

Figure 10. Learning performance in the 5-Choice Reaction Time task in EXP1 (A) and EXP2 (B). Learning curves of control and STZ-treated animals are depicted. Shaded areas show the 95% confidence band of the fitted sigmoidal regression curves (thin [red] lines). The column chart inset shows the number of days taken to reach the maximum stage. Means ± SEM values are shown. In EXP2, the regression lines significantly differ (no overlap between the confidence bands) (47).

Western blot measurements

In EXP1, western blot analysis revealed no significant difference in phospho-tau/tau ratio and β-amyloid level between vehicle- and STZ-treated animals (Fig. 11A and 11C). In EXP2 we found a marginally significant elevated phospho-tau/tau ratio (Fig. 11B) and a significant increase in the β-amyloid level in the STZ-treated animals (Fig. 11D) (47).

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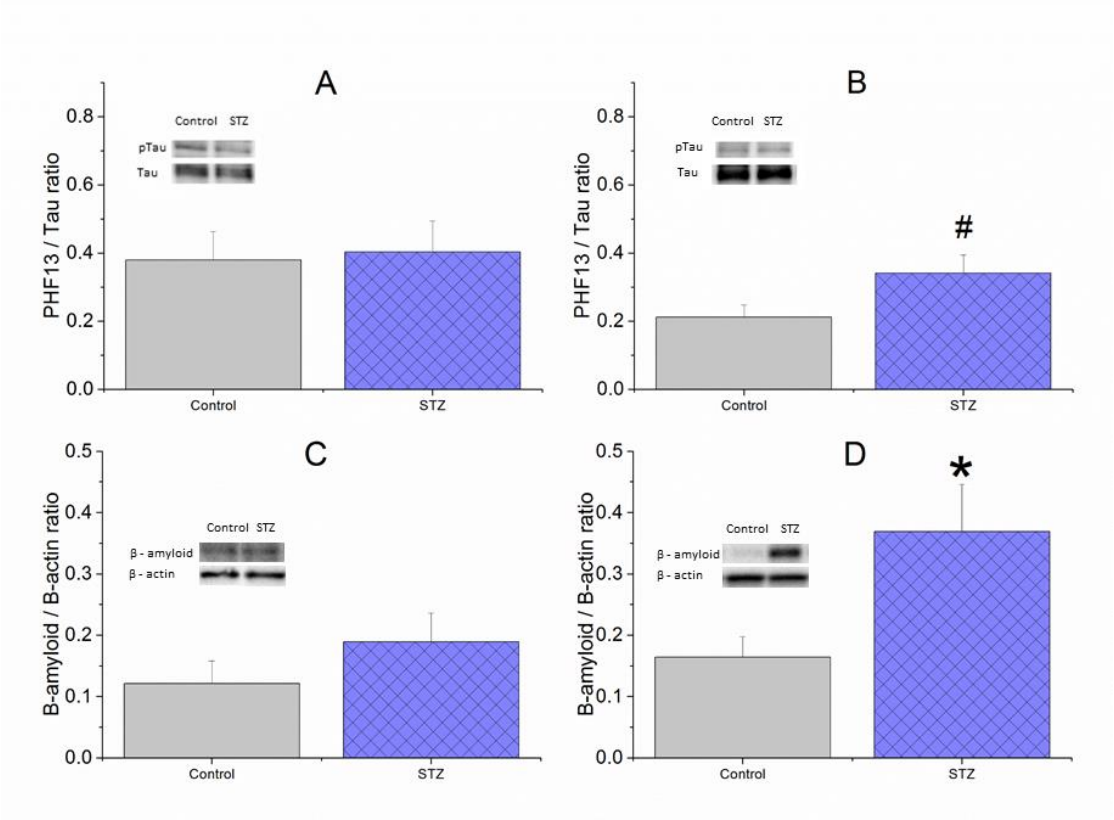

Figure 11. The effect of icv. STZ or vehicle (control) treatment on the tissue protein levels of phospho-Tau (A and B) and β-amyloid (C and D) in EXP1 (A and C) and EXP2 (B and D), measured by western blot. Means ± SEM values are shown. #: $p<0.06$ (unpaired t-test: $t(22)= -2.012$); *: $p<0.05$ (unpaired t-test: $t(20)= -2.45$) (47).

Multivariate analysis of variance

We found more pronounced effects in four out of the six assays in EXP2 but not in EXP1, although in themselves, they were not always statistically significant. To statistically analyse the overall difference between the two experimental groups we performed a multivariate ANOVA on four variables each from one of these 4 assays: phospho-tau/tau ratio, β-amyloid level, NOR discrimination index, and days needed to reach the final stage in the 5-CSRTT. The difference between the control and STZ groups was significant in EXP2 (Wilks $\lambda=0.397$, $F(4,13)=4.931$; $p=0.012$) whereas it was not significant in EXP1 (Wilks $\lambda=0.583$, $F(4,6)=1.072$; $p=0.446$) (47).

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Short summary[1]

In order to reproduce in naive Long-Evans rats the cognitive and biochemical changes described in Wistar rats in the literature, in our first experiment (EXP1) we chose the widely used dose of 2x1.5 mg/kg icv. STZ to evoke cognitive deficits. During EXP1, we couldn't find any 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. STZ treated animals acquired the MWM and 5-CSRTT tasks as well as their controls. In the PAL test relatively low memory trace could be observed even in the control group. In the NOR assay the control animals showed a sufficient level of discrimination while the STZ-treated rats were much inferior, but due to the low final sample size these differences were not significant. The outcomes from EXP1 implied that the dosage of STZ may not have been sufficient for Long-Evans rats (47).

Therefore, in a subsequent study (EXP2) we increased the dose of the icv. STZ (3x 1.5 mg/kg) and also changed the timing of cognitive assays. In EXP2, the effects of the STZ treatment were more pronounced in the NOR, 5-CSRTT, β-amyloid, and phospho-tau assays compared to EXP1, which was confirmed by the multivariate analysis. However, we observed no difference in the MWM and PAL tests. Our results suggest that Long-Evans rats may not be as affected by STZ treatment as albino rat strains, and indicate that the 3 × 1.5 mg/kg dose was sufficient to evoke biochemical changes (47).

4.2. Study in young experienced animals

Based on the previous experiments, the examination of the effects of icv. STZ on young experienced animals was continued with the dose of 3x1.5 mg. Twenty-four 10 months 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 the surgeries,

[1] In order to make the thesis easier to read and follow, we have inserted a summary at the end of each Results subsection. The texts of these summaries are by large part taken from the articles that contain the results, referred at the end of the paragraph.

the animals were re-tested in the learnt paradigms to see the – possibly time dependent - effect of the STZ treatment. To examine the effect of STZ treatment on visual recognition memory and on aversive learning additional tasks were introduced, such as NOR for recognition memory, PAL and fear conditioning (FC) for fear memory. 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 (Fig. 12) (46).

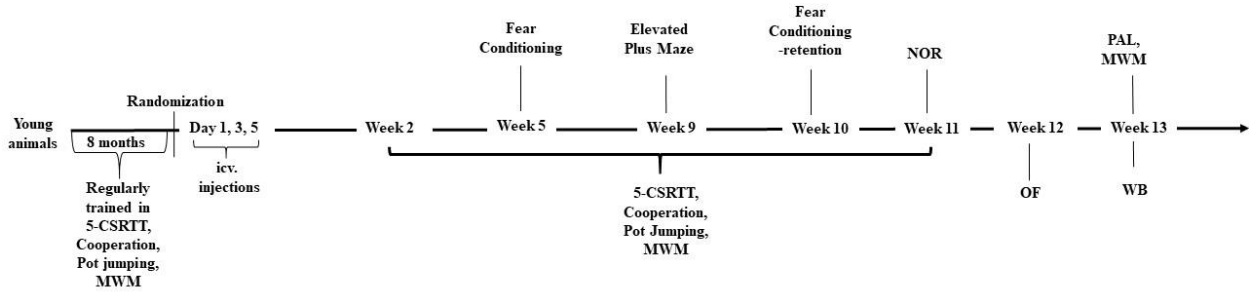

Figure 12. Timeline of the experiments in young experienced animals (icv: intracerebroventricular; STZ: streptozotocin; NOR: novel object recognition; 5-CSRTT: five choice serial reaction time task; OF: open field; PAL: passive avoidance learning; MWM: Morris water-maze; WB: western blot) (46).

Novel object recognition (NOR)

We found significant difference between the groups in the DI parameters. STZ-treated animals had a significantly lower DI (0.05) compared to the controls (0.25) (Fig. 13) (46).

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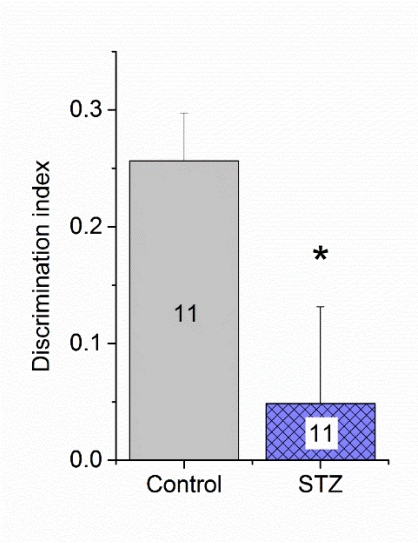

Figure 13. Novel object recognition performance of icv. STZ-injected (STZ) and vehicle treated (control) rats at Week 11 post-injection. Columns show means ± SEM values of discrimination index. Numbers inside the columns indicate the number of animals. *: $p<0.05$ vs control, unpaired t-test, $t(20) = 2.24$. Two rats were excluded from the evaluation according to the criteria described in chapter 4.1. The inter-trial period was 60 min (46).

Passive avoidance learning (PAL)

During the acquisition trial, the STZ-treated animals showed significantly longer latency to enter the dark chamber compared to the controls. In turn, there was no significant difference between the groups in the retention trial (Table 1) (46).

Table 1. Results of icv. STZ-injected (STZ) and vehicle-treated (control) rats in passive avoidance learning test at Week 13 post-injection. *: $p<0.05$ significant difference vs control; +++: $p<0.001$ significant difference vs acquisition trial (post-hoc Duncan-test following repeated measures ANOVA with significant 'treatment' ($F(1,20)=5.41$, $p=0.030$) and 'trial' ($F(1,20)=397.41$, $p=0.000$)) effects. Group size of STZ-treated rats: n= 10 (46).

Test	Control		STZ	
	Mean	±*SEM*	Mean	±*SEM*
PAL acquisition trial entry latency (s)	45.7	*±9.92*	88.6^{*}	*±13.91*
PAL retention trial entry latency (s)	278.4^{+++}	*±17.07*	300^{+++}	*±0*
Not entered/total number of animals	10/12		10/10	

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Morris water-maze (MWM)

STZ-treated rats needed significantly longer time to find the hidden platform. The difference was maintained throughout the whole measurement period except at week 13, when the treated animals performed similarly to controls though still significantly worse than at their own baseline (Fig. 14) (46).

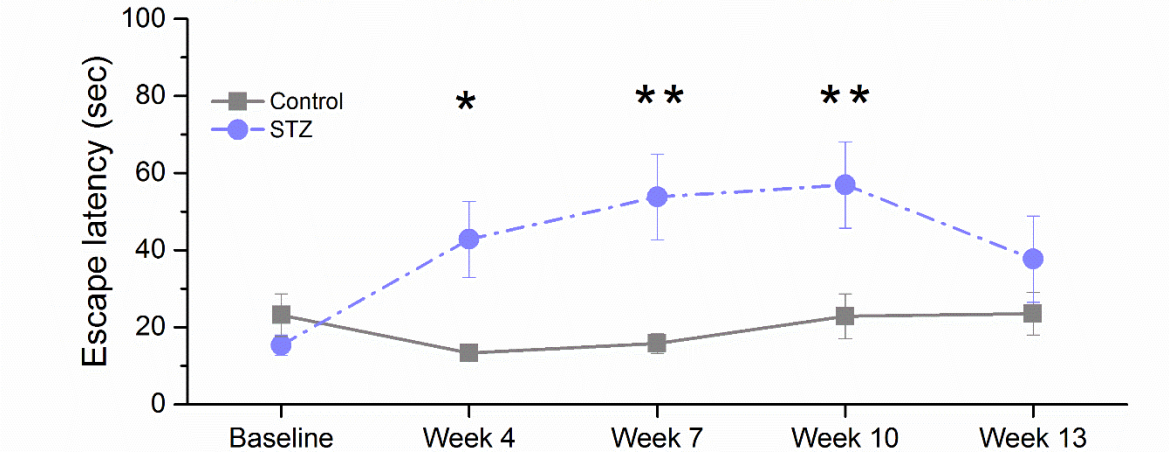

Figure 14. Learning performance of icv. STZ-injected (STZ) and vehicle-treated (control) young rats in the Morris water-maze at various time points post-injection. Each measurement point represents the result of a single daily session consisting of 3 trials. Group means ± SEM of individual average daily latency values are shown. Platform location was changed at each measurement. *, **: $p<0.05$, $p<0.01$: significant difference between groups on weeks 4, 7 and 10 (post-hoc Duncan test following repeated measures ANOVA with significant week × treatment interaction: $F(4,88) = 3.88$, $p<0.01$) (46).

5-choice serial reaction time task (5-CSRTT)

STZ-treated animals showed significantly reduced correct responses and increased omissions (when the rat did not make any nose-poke in response to the light stimulus) up to Week 6 (Fig. 15A-B) with preserved accuracy $\left(\frac{total\ correct\ asnwers}{total\ correct\ answers + total\ incorrect\ answers} x100\right)$ (Fig. 15D) and unchanged premature nosepokes (nose-poked into any of the holes during the inter-trial interval) (46).

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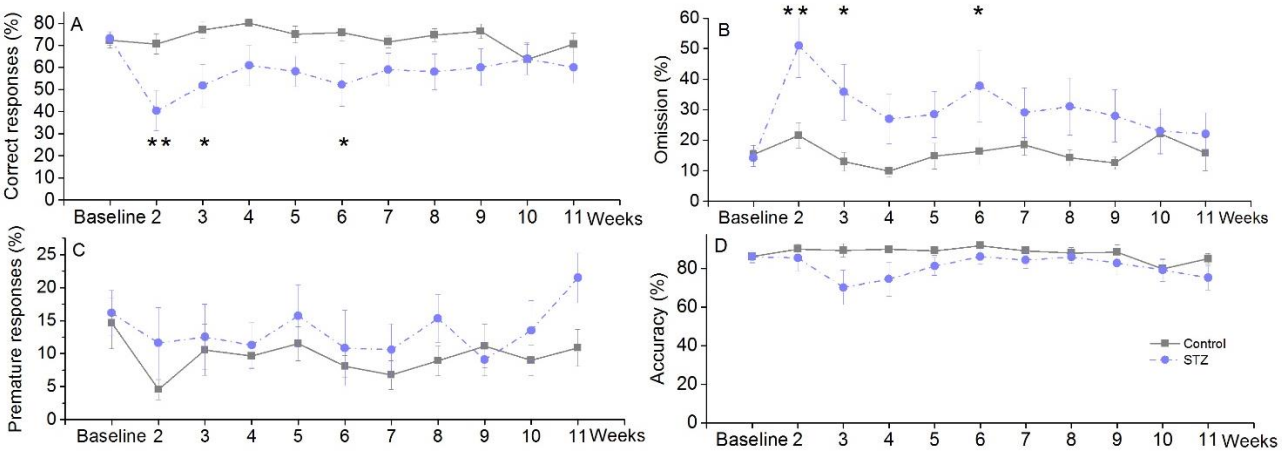

Figure 15. Learning performance of icv. STZ-injected (STZ) and vehicle-treated (control) rats in the 5-CSRTT at various time points post-injection. A: % correct responses, B: % omissions, C: 5 premature responses, D: accuracy. Means ± SEM values are shown.*, **: $p<0.05$, $p<0.01$ significant difference between groups on the same day (post-hoc Duncan test following repeated measures ANOVA with treatment effect: $F(1,22) = 5.18$, $p<0.05$ and week × treatment interaction: $F(10,220) = 2.20$, $p<0.05$ for percentage of correct responses (A) and treatment effect: $F(1,22) = 3.94$, $p < 0.06$ and week × treatment interaction: $F(10,220) = 2.06$, $p<0.05$ for omissions) (46).

Pot jumping test

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 (46).

STZ-injected rats jumped over significantly shorter distance (about one pot difference) than control rats, and made significantly less passes between the pots at the first post-treatment occasion (Week 2) (Fig. 16A, 4B) (46).

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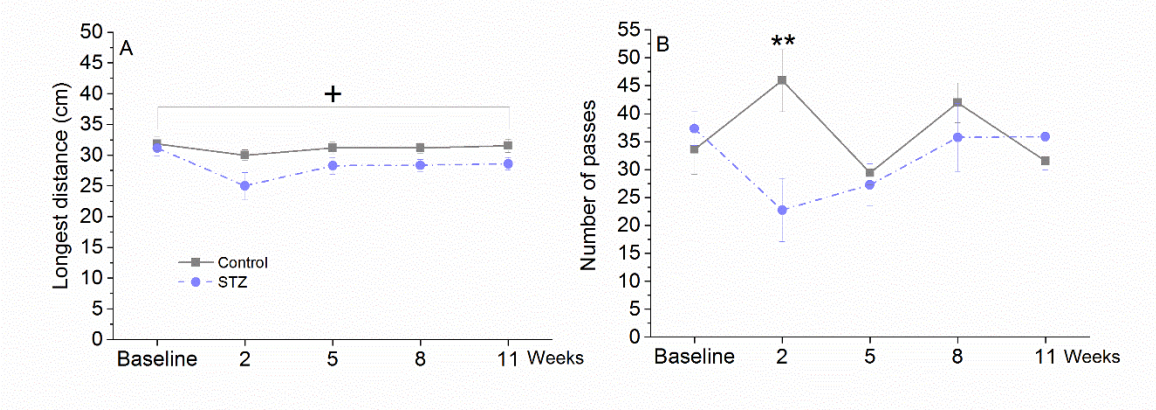

Figure 16. Performance of icv. STZ-injected (STZ) and vehicle-treated (control) rats in the pot jumping task at various time points post-injection. Means ± SEM of longest distance jumped over (A) and number of passes (B) are shown. +: $p<0.05$ significant treatment effect ($F(1,22) = 4.42$). **: $p<0.01$ significant difference between groups on the same day (post-hoc Duncan test following repeated measures ANOVA with significant Day × treatment interaction: $F(4,88) = 5.20$, $p<0.001$ (46).

Cooperation task

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 nose poke 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 (46).

ANOVA did not reveal any significant effect either for the treatment or the repeated sessions or their interaction (Fig. 17). Note, however, the decrease in performance in both groups at the first occasion after the surgery (46).

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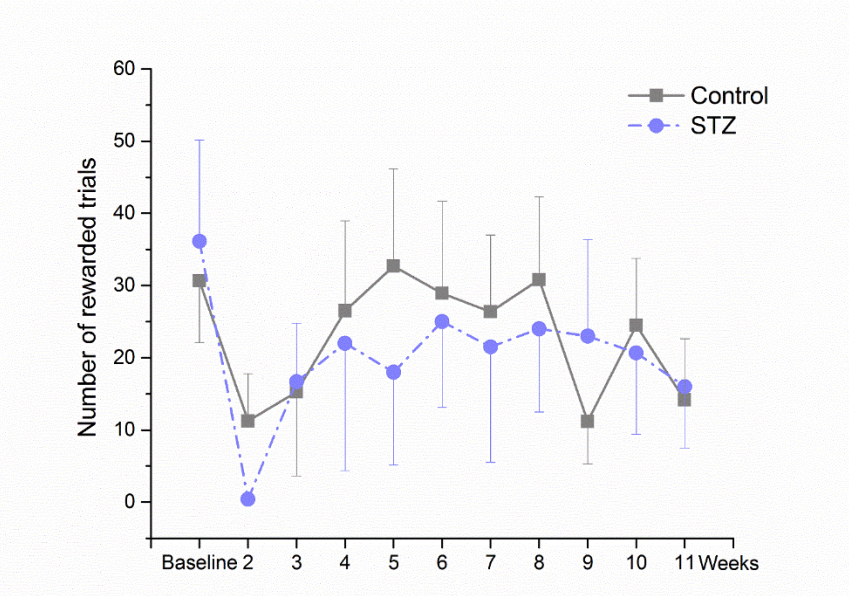

Figure 17. Learning performance of icv. STZ-injected (STZ) and vehicle-treated (control) rats in a cooperation task at various time points post-injection. Means ± SEM of number of rewarded trials are shown. n=6 pairs in each group (46).

Fear conditioning (FC)

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 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 (46).

There was no significant difference between the behaviour of the animals either in acquisition trials, or in retention trials (Table 2) (46).

Table 2. Results of icv. STZ-injected (STZ) and vehicle-treated (Control) rats in the fear conditioning paradigm at Week 5 and 10 post-injection. +++: p<0.001 significant difference vs acquisition trial (post-hoc Duncan-test following repeated measures ANOVA with significant 'trial' effect (F(2,44)=43.54, p<0.001) (46).

Test	Control		STZ	
	Mean	±*SEM*	Mean	±*SEM*
FC acquisition trial freezing time (s)	123.0	*±11.95*	84.9	*±14.22*
FC retention trial freezing time 24h (s)	204.5^{+++}	*±21.02*	208.4^{+++}	*±22.15*
FC retention trial freezing time 1 months (s)	187.0^{+++}	*±25.74*	190.3^{+++}	*±22.74*

Elevated plus maze (EPM)

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. Animals which did not moved from the central square were excluded from the experiment (46).

There was no significant difference between the two groups in either of the variables (Table 3) (46).

Table 3. Results of icv. STZ-injected (STZ) and vehicle-treated (control) rats in the elevated plus maze at Week 9 post-injection. Group size of young STZ-treated rats: n= 11 (46).

Test	Control		STZ	
	Mean	±*SEM*	Mean	±*SEM*
EPM time spent in open arms (s)	19.4	*±8.40*	12.2	*±5.0*
EPM percentage of open/total entries	19	*±0.063*	29	*±0.085*

Open field (OF)

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. Analysed variables were the ambulation time, local movement time and immobility time (46).

STZ-treated rats demonstrated significantly increased activity. Consequently, they spent significantly less time in immobility (Table 4) (46).

Table 4. Open field results of icv. STZ-injected (STZ) and vehicle-treated (control) rats at Week 12 post-injection. Columns include means ± SEM values. **, ***: $p<0.01$, $p<0.001$ significant difference between groups; unpaired t-test, ambulation time: $t(22) = -3.11$, local movement time: $t(22) = -4.05$, immobility time: $t(22) = 3.05$ (46).

Test	Control		STZ	
	Mean	±*SEM*	Mean	±*SEM*
Ambulation time	332.6	*±13.67*	409.2**	*±20.48*
Local movement time	645.6	*±15.43*	758.5***	*±23.12*
Immobility time	670.1	*±24.79*	541.4**	*±34.13*

Phospho-tau and beta-amyloid levels

We detected a non-significant, moderate increase in phospho-tau/tau ratio in STZ-treated rats compared to their respective controls whereas no difference was found in β-amyloid levels between STZ-treated and control groups (Table 5) (46).

Table 5. Results of the western blot assays at Week 13 post-injection (46).

Test	Control		STZ	
	Mean	±*SEM*	Mean	±*SEM*
phospho-tau / tau	0.54	*±0.09*	0.91	*±0.21*
β-amyloid / β-actin	0.5	*±0.08*	0.5	*±0.10*

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Short summary[2]

Based on the previous findings, we continued the experiments with the 3x1.5mg/kg dose of icv. STZ. In young experienced rats, STZ-treatment impaired recognition memory (NOR), spatial memory (MWM) and attention (5-CSRTT). 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 (pot jump test) 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, which suggests STZ treatment did not affect fear memory and social learning. 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 null change could be observed while in the latter a non-significant, moderate increase was detected (46).

4.3. Study in old experienced animals

Based on the previous experiments, the examination of the effects of the icv. STZ on aged experienced animals were also performed with the dose of 3x1.5 mg. Twenty-nine 23 months old male Long-Evans rats were used in this study. The animals had had a long learning history in several paradigms for 21 months: 5-CSRTT for attention, a cooperation task for social cognition, MWM paradigm for spatial memory, “pot-jumping” exercise for procedural memory. After the 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 to examine the effect of STZ treatment on visual recognition memory and on aversive learning such as NOR for recognition memory, PAL and fear conditioning for fear memory. To examine the ability to acquire new knowledge, pairwise visual discrimination learning was tested for 9 days. Besides, open-field

[2] In order to make the thesis easier to read and follow, we have inserted a summary text at the end of each Results subsection. The texts of these summaries are by large part taken from the articles that contain the results, referred at the end of the paragraph.

behaviour for measuring basic spontaneous motor activity and EPM performance for measuring anxiety were also examined. At the end of the behavioural measurements the animals were sacrificed, their hippocampi were harvested for western blot measurements. The experiments lasted for 15 weeks (Fig. 18). Unfortunately, two STZ-injected and three control rats did not recover from the surgical anaesthesia. We lost four additional animals from the STZ group during the course of the experiment. Two died at weeks 2 and 11, while two others were euthanized due to poor health at weeks 9 and 11. The control group size remained 12 during the post-surgery period (46).

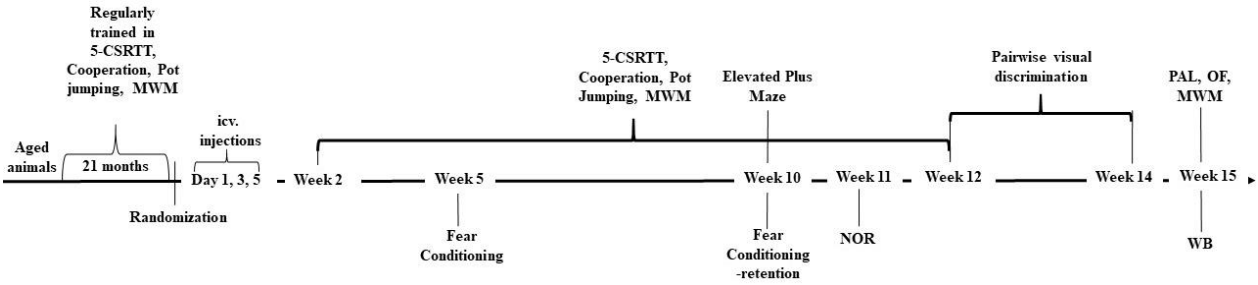

Figure 18. Timeline of the experiments in old experienced animals (icv: intracerebroventricular; STZ: streptozotocin; NOR: novel object recognition; 5-CSRTT: five choice serial reaction time task; OF: open field; PAL: passive avoidance learning; MWM: Morris water-maze; PD: pairwise visual discrimination; WB: western blot) (46).

Novel object recognition (NOR)

We did not find significant difference between the groups in the DI parameters. However, control animals showed a DI (0.19) significantly different from zero, whereas the DI of STZ-treated rats (0.06) did not differ from zero (meaning no discrimination) (Fig. 19) (46).

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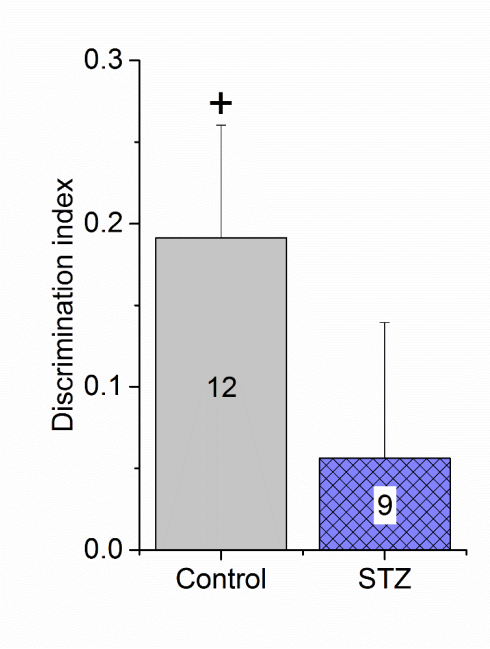

Figure 19. Novel object recognition performance of icv. STZ-injected (STZ) and vehicle treated (control) rats at Week 11 post-injection. Columns show means ± SEM values of discrimination index. Numbers inside the columns indicate the number of animals. +: $p<0.05$ vs zero, singe sample t-test, control: $t(11) = 2.76$, STZ: $t(8) = 0.67$, ns. (46).

Passive avoidance learning (PAL)

There was no significant difference between the learning performances of groups either in acquisition or retention trials (Table 6) (46).

Table 6. Results of icv. STZ-injected (STZ) and vehicle-treated (control) rats in passive avoidance learning test at Week 15 post-injection. +++: $p<0.001$ significant difference vs acquisition trial (post-hoc Duncan-test following repeated measures ANOVA with significant 'trial' effect ($F(1,18)=89.44$, $p<0.001$). Group size of STZ-treated rats: n= 8 (46).

Test	Control		STZ	
	Mean	±*SEM*	Mean	±*SEM*
PAL acquisition trial entry latency (s)	33.5	*±8.23*	33.3	*±2.86*
PAL retention trial entry latency (s)	226.1^{+++}	*±35.06*	273.8^{+++}	*±7.32*
Not entered/total number of animals	8/12		6/8	

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Morris water-maze (MWM)

STZ-treated rats needed significantly longer time to find the hidden platform (Fig. 20) (46).

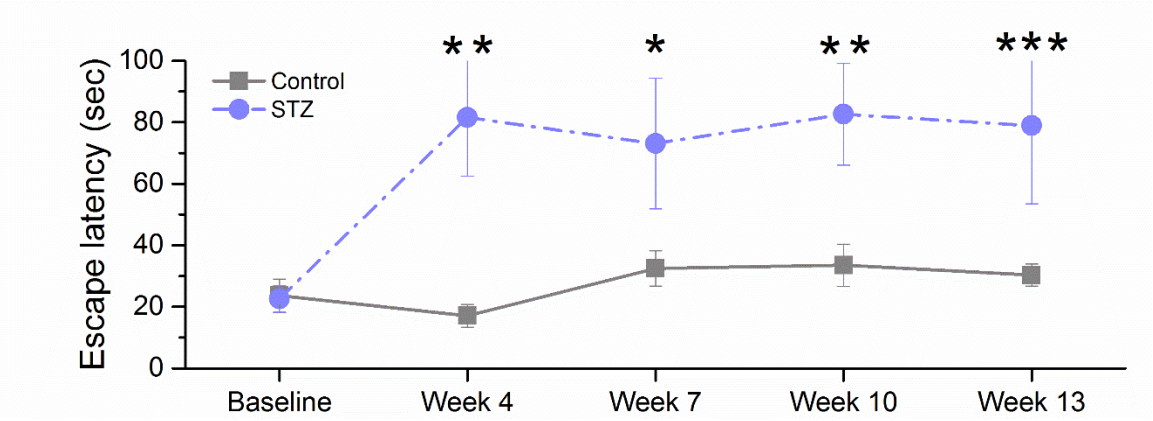

Figure 20. Learning performance of icv. STZ-injected (STZ) and vehicle-treated (control) rats in the Morris water-maze at various time points post-injection. Each measurement point represents the result of a single daily session consisting of 3 trials. Group means ± SEM of individual daily average latency values are shown. *, **, ***: $p<0.05$, $p<0.01$ $p<0.001$: significant difference between groups on days 1 2, 3 and 4 (post-hoc Duncan test following repeated measures ANOVA with significant treatment effect: $F(1,21)=10.38$, $p<0.001$, and week × treatment interaction: $F(4,84) = 6.13$, $p<0.001$). Group size of old STZ-treated rats: n= 11 at Week 4 and 6-8, n=10 at Week 9-11, n=8 at Week 14-15 (46).

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5-choice serial reaction time task (5-CSRTT)

In this study no significant difference was found between the groups in the percentage of correct responses and omissions (Fig. 21A-B). STZ-treated rats produced significantly more premature responses than that of the controls in the post-injection period from Week 2 to Week 12 (Fig. 21C). Response accuracy was significantly lower in the STZ-treated group on the first post treatment occasion, however, this difference was not detectable on subsequent measurement days (Fig. 21D) (46).

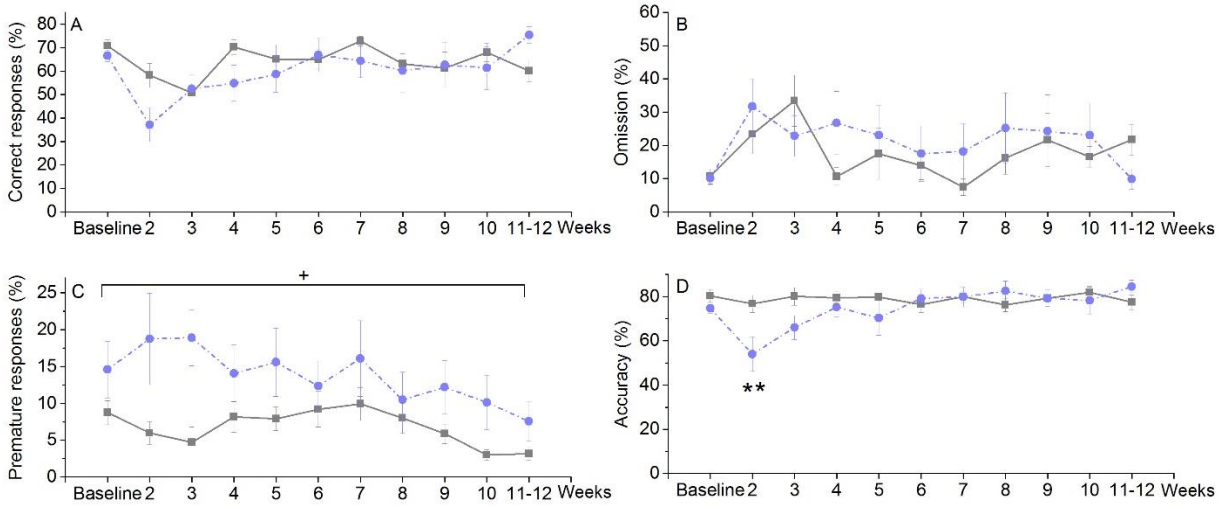

Figure 21. Learning performance of icv. STZ-injected (STZ) and vehicle-treated (control) rats in the 5-CSRTT at various time points post-injection. Means ± SEM values are shown. A: % correct responses. B: % omissions. C: % premature responses. +: $p<0.05$ significant treatment effect ($F(1,21) = 5.98$). D: Accuracy. **: $p<0.01$ significant difference vs control on the same day (post-hoc Duncan test following repeated measures ANOVA with significant week × treatment interaction: $F(10, 200)=2.53$, $p<0.01$). Group size of old STZ-treated rats: n= 12 at Week 2, n= 11 at Week 3-9, n=10 at Week 10, n=8 at Week 11-12 (46).

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Pot jumping test

We could not detect significant difference between the groups in this procedural learning task either in the longest interpot distance jumped over or in the number of passes (Fig. 22) (46).

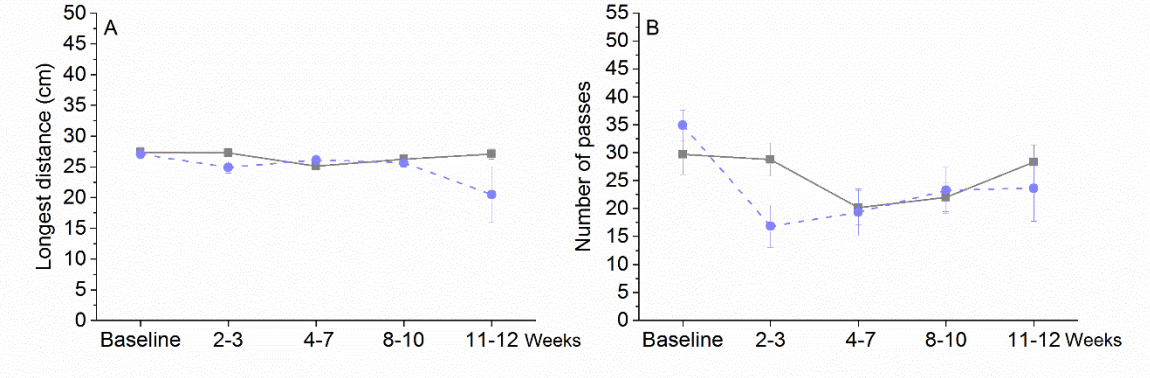

Figure 22. Performance of icv. STZ-injected (STZ) and vehicle-treated (control) rats in the pot jumping task at various time points post-injection. Means ± SEM of longest distance jumped over (A) and number of passes (B) are shown. Group size of old STZ-treated rats: n = 11 at Week 2–3, 4–7 and 8–10, n = 8 at Week 11–12 (46).

Cooperation

Because of the high mortality rate, the pairs were broken and it was not possible to evaluate the data. We tried to put the unpaired animals in the same group together, but only two newly formed pairs started to work, therefore the results of this task could not be evaluated (46).

Fear conditioning (FC)

No significant difference was found between the behaviour of the animals in acquisition trial. STZ-treated animals had longer freezing time compared to the controls in the retention trials (24 h and 1 month later) but the difference was only marginally significant (repeated measures ANOVA, treatment effect: $F(1,20)=4.08$, $p=0.057$; treatment x trial interaction: $F(2,40)=3.06$, $p=0.058$) (Table 7) (46).

Table 7. Learning performance of icv. STZ-injected (STZ) and vehicle-treated (control) rats in the fear conditioning paradigm at Week 5 and 10 post-injection. +++: $p<0.001$ significant difference vs acquisition trial (post-hoc Duncan-test following repeated measures ANOVA with significant 'trial' effect ($F(2,40)=18.36$, $p<0.001$). Group size of old STZ-treated rats: n= 11 at retention trial 24h and n=10 at 1 month. Group size of old STZ-treated rats: n=10 (46).

Test	Control		STZ	
	Mean	±*SEM*	Mean	±*SEM*
FC acquisition trial freezing time (s)	31.7	*±14.39*	43.3	*±11.24*
FC retention trial freezing time 24h (s)	76.9^{+++}	*±21.63*	161.2^{+++}	*±30.52*
FC retention trial freezing time 1 months (s)	89.0^{+++}	*±25.22*	168.3^{+++}	*±32.49*

Elevated plus maze (EPM)

STZ-treated animals spent more time in the open arms and the ratio of open/total entries was significantly larger compared to the controls (Table 8) (46).

Table 8. Results of icv. STZ-injected (STZ) and vehicle-treated (control) rats in elevated plus maze test at Week 10 post-injection. § $p=0.042$ significant difference vs control (Mann-Whitney U-test, $U=29$; because of variance inhomogeneity non-parametric test was used). Group size of old STZ-treated rats: n= 10 (46).

Test	Control		STZ	
	Mean	±*SEM*	Mean	±*SEM*
EPM time spent in open arms (s)	5.3	*±3.25*	34.1	*±17.98*
EPM percentage of open/total entries	3.7	*±0.022*	$21^{\S}$	*±0.095*

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Open field (OF)

STZ-treated rats demonstrated significantly increased activity. Consequently, they spent significantly less time in immobility (Table 9) (46).

Table 9. Open field results of icv. STZ-injected (STZ) and vehicle-treated (control) rats at Week 15 post-injection. Columns include means ± SEM values. *: $p<0.05$ significant difference between groups, unpaired t-test, ambulation time: $t(18) = -2.32$, local movement time: $t(18) = -2.72$, immobility time: $t(18) = 2.72$. Group size of old STZ-treated rats: n=8 (46).

Test	Control		STZ	
	Mean	±*SEM*	Mean	±*SEM*
Ambulation time	188.4	±*23.23*	268.5*	±*23.62*
Local movement time	727.9	±*34.40*	871.3*	±*38.34*
Immobility time	833.4	±*55.32*	617.8*	±*49.67*

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) (46).

STZ-treated animals made a significantly higher number of incorrect responses (Fig. 23B) and their number of completed trials were also significantly higher compared to the controls (Fig. 23D). The number of correct responses also increased, though it was not statistically significant. There was no difference between the two groups in the percentage of correct responses (Fig. 23A, C) (46).

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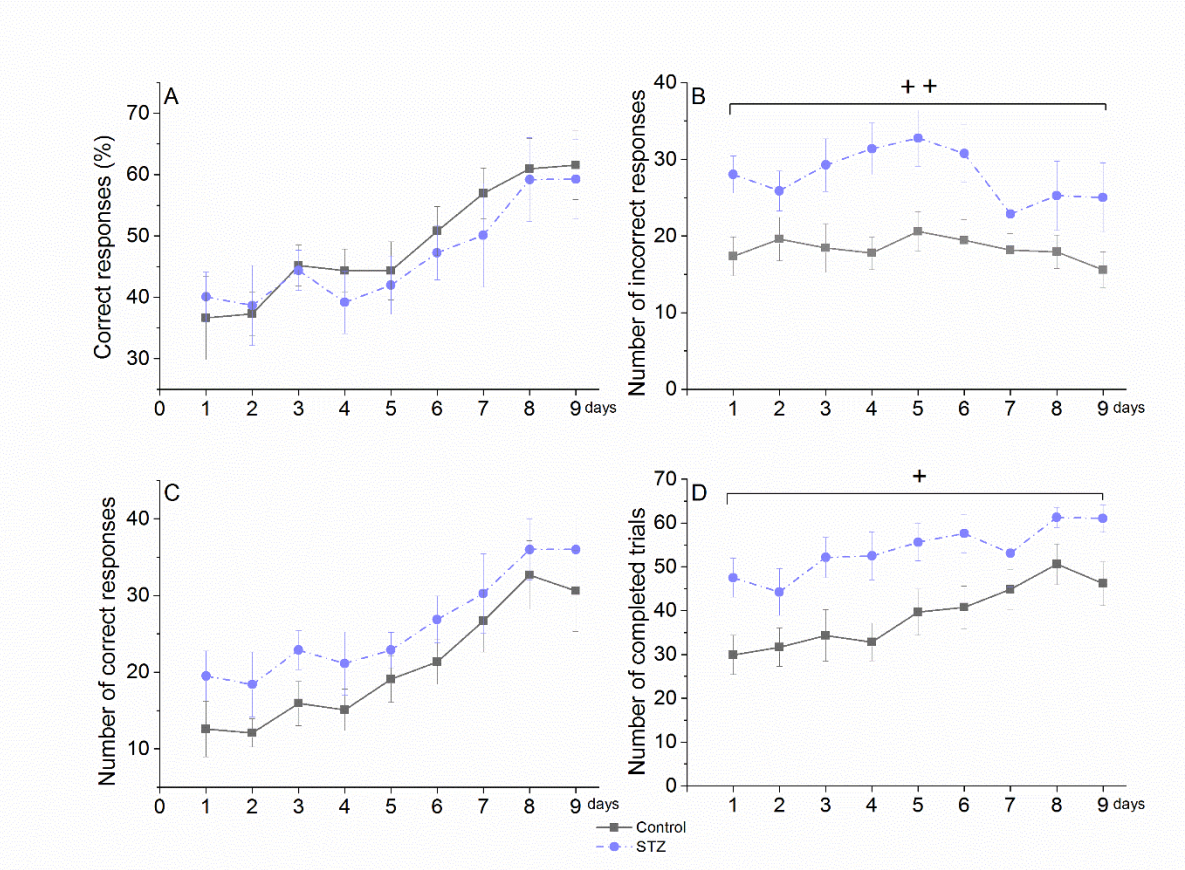

Figure 23. Pairwise visual discrimination learning curves of icv. STZ-injected (STZ) and vehicle-treated (control) rats in a touchscreen apparatus in the post-injection period of Week 12-14. Means ± SEM values are shown. A: percentage of correct responses. B: number of incorrect responses. ++: $p<0.01$ significant treatment effect, $F(1,18)=10.28$. C: number of correct responses. D: number of completed trials. +: $p<0.05$ significant treatment effect ($F(1,18) = 6.83$). Group size of old STZ-treated rats: n= 8 (46).

Phospho-tau and beta-amyloid levels

Significant elevated phospho-tau/tau ratio was found in old STZ-treated rats compared to their respective controls, while no difference was observed in β -amyloid levels between STZ-treated and control groups (Table 10) (46).

Table 10. Results of the western blot assays at Week 15 post-injection. Phospho-tau/tau ratio, §: p=0.016 significant difference vs control (Mann-Whitney U-test U=18; because of variance inhomogeneity non-parametric test was used), effect size: 1.23. Group size of old STZ-treated rats: n= 9 (phospho-tau/tau ratio), n=10 (β-amyloid level) (46).

Test	Control		STZ	
	Mean	±*SEM*	Mean	±*SEM*
phospho-tau / tau	0.22	*±0.03*	0.43§	*±0.08*
β-amyloid / β-actin	0.2	*±0.03*	0.2	*±0.05*

Short summary[3]

An important finding of the study was that 3x1.5 mg/kg STZ was toxic to the old animals, as we lost four drug-treated rats during the post-treatment period. The treatment impaired recognition (NOR) and spatial memory (MWM) 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. During 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 untreated rats. 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-

[3] In order to make the thesis easier to read and follow, we have inserted a summary text at the end of each Results subsection. The texts of these summaries are by large part taken from the articles that contain the results, referred at the end of the paragraph.

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 (46).

4.4. Age dependence of β-amyloid level

To test the assumption that the lack of increased β-amyloid level may have been due to a ceiling effect, in a separate measurement we re-assayed the tissue protein levels of β–amyloid in the control rats of all the three studies. Thus, we compared 5.5-month-old (naïve), 13-month-old (experienced) and 27-month-old (experienced) rats (46).

We found an age-dependent increase in β-amyloid level with significant differences between the three age groups (Fig. 24) (46).

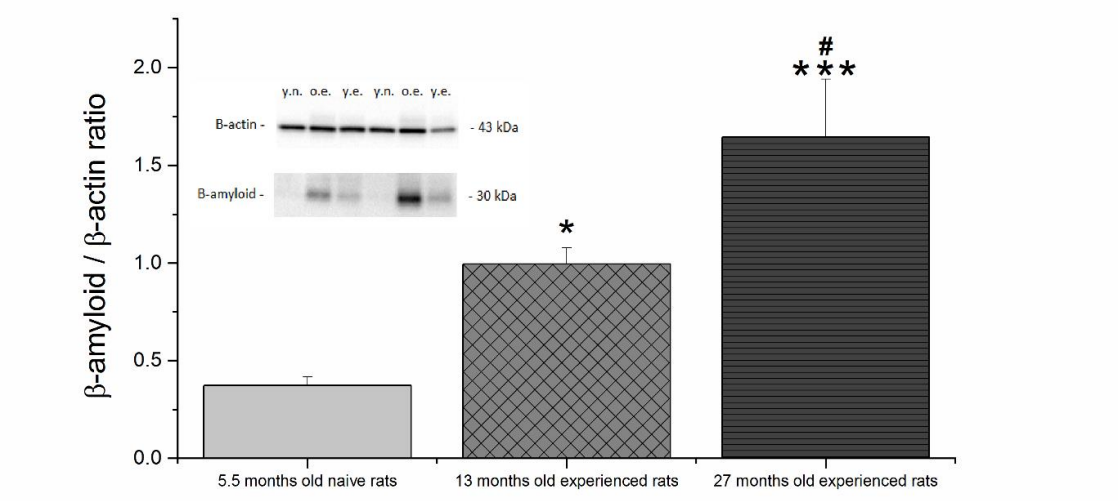

Figure 24. Comparison of tissue protein levels of β-amyloid in 5.5-month-old (young naïve), 13-month-old (young experienced) and 27-month-old (old experienced) rats measured by western blot. Means ± SEM values are shown. *, ***: $p<0.05$, $p<0.001$ significant difference vs. young naïve rats, #: $p<0.05$ significant difference vs. young experienced rats (post-hoc Duncan test following one way ANOVA ($F(2,24) = 10.09$, $p<0.001$). Group sizes are 9, 11 and 11 for young naïve (y.n.), young experienced (y.e.) and old experienced rats (o.e.), respectively. The inset shows representative blots (46).

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5. Discussion

Comparing the results obtained from the three STZ-treated groups, similar findings were obtained in some tests while different results in others, both in relation to each other (Table 11) and to the literature.

Table 11. 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
Spontaneous motor activity	x	Increased	Increased
Impulsivity / motivation	x	Not observed	Increased
Phospho-tau	Increased	Increased	Increased
β-amyloid	Increased	Unchanged	Unchanged

In all the three experiments we found an impairment in recognition memory, as all treated animals performed worse in the NOR test compared to their controls. Impaired recognition memory in the NOR test is a common finding in the literature (47,72,81,89,99,100,102). Interestingly, Fine et al.

(67) found no difference between the STZ treated and control groups in naïve Long-Evans rats, although compared to our measurements (which were carried out at week 8, 11 or 14 post injection), they ran the test at a different time period (between week 4 and 7). Furthermore, Silva et al. (85) found impaired recognition memory after 30 days but not after 120 days in Wistar rats. These results may suggest the potential time-dependent effectiveness of the treatment, but it is worth making some reservation in connection with NOR results, since the sensitivity of the test is heavily influenced by the animal's interest in the objects.

Impaired spatial learning was found in the young and old experienced rats. Decreased spatial learning in the MWM is one of the most common and characteristic effects of STZ experiments (57–59,69–71,73–75,82,84,86,88,91,92,97,98,100,101). The papers of Majkutewitz et al. (58), Wrona et al (59) and Kurowska-Rucinska et al. (57) are of particular relevance in this comparison as the authors – similarly to us - examined 22-month-old (Wistar) rats and applied a protocol where the platform location changed day by day.

However, in young naïve Long-Evans rats we did not find impaired MWM learning. It is in contrast to the majority of the Wistar literature and also to studies that used young Long-Evans rats (61,64,67). Although in some of the MWM studies difference between the control and the STZ treated rats was only found in the probe trial (75,76,127), we could observe no difference even there. Unfortunately, we cannot give a plausible explanation for this discrepancy, unless we assume our result is a sporadic 'outlier" in the STZ literature, where conflicting results are not uncommon (see below).

The animals' procedural memory was examined only in the case of trained animals. Impaired procedural memory was found in young but not in old rats (presumably because of a floor effect). Regarding the effect of STZ on procedural learning, the literature is controversial. Impaired motor learning was found in young naïve Long-Evans rats in rotarod and balance beam tests (65,67), but not in young naïve Wistar rats in rotarod test (128,129) and balance beam test (92). The results suggest that the icv. STZ evoked procedural memory impairment may be strain dependent.

Impaired attention in 5-CSRTT was found in naive and trained young rats (however, the latter effect was transient, as it passed by the end of the experiment) but not in old animals. These results suggest that the previously acquired knowledge could compensate the detrimental effect. To the

best of our knowledge, this was the first time that attention has been studied in icv. STZ treated animals.

Social memory was only tested in the young experienced rats (due to the high mortality rate, it was not possible to evaluate data in the old experienced animals). The treatment has no effect on social memory in young trained animals. Similar to the previous paradigm, this was the first time that social memory has been studied in icv. STZ treated animals.

To examine the ability to acquire new knowledge, visual discrimination learning was tested in old experienced rats. Although, visual discrimination itself was not affected (there was no difference in the % correct responses), the number of completed trials and the number of incorrect responses significantly increased. We could not find studies on pairwise visual recognition in icv. STZ treated rats in the literature.

When testing fear memory, even though PAL impairment is one the most common findings in the icv. STZ literature (43,68–70,72,73,78,80,84,88,95,96,99), in neither study we could detect changes in this assay. Interestingly, in a very recent study, the authors – in contrast to their earlier findings (43,69,73) – did not find impairment in the PAL test (130). In the case of trained animals, we also examined fear memory with the FC test. A marginally-significant difference was found in old (but not in young) trained animals: treated rats had a longer freezing time compared to the controls. We found only one FC study in the literature (81), which reported decreased freezing response in the tone-conditioned but not in the context-conditioned version of the test in young naïve icv. STZ-injected Wistar rats. These contrasting findings may again indicate strain dependence.

Increased activity was found in OF test in both experiments with trained animals (naïve animals were not tested), which was associated with reduced anxiety in the EPM in old animals. In the literature, OF measurements were only carried out in young rats, with one exception. Our findings in the open-field test are similar to (55,67,127) but in contrast to (68,70,78,95,96) who did not find difference in this test. The only study which examined old rats in the open field, also did not find differences in basic activity, but STZ-treated rats spent more time in the center part of the open-field (56). In the literature, anxiety level in the EPM was only measured in young naïve STZ-treated animals. Two studies, Ileva et al. (93) and Roy et al. (83) observed – in contrast to our results – increased anxiety, while Moreira-Silva et al. (81) found no difference from the control. However,

the above cited OF finding of increased time in the center zone in old animals (56) also can be interpreted as reduced anxiety, thus supporting our results in the EPM.

Elevated premature responses in the 5-CSRTT and increased incorrect responses in the visual discrimination test were found in the case of aged animals. The former is consensually interpreted in the literature as a sign impulsivity (110). Together with the increased open-field activity, reduced anxiety and the observed differences in the FC test, these results suggest that beside its cognitive effects icv. STZ exerted emotional effects as well. Elevated impulsivity would explain the seemingly contradictory results of the FC (increased anxiety) and EPM (decreased anxiety) tests, since both responses may indicate an impulsive overreaction to the actual situation. The animals showed more courageous exploration in the EPM while more fearful behaviour in the FC test. The increased number of initiated trials by the STZ treated animals in the pairwise discrimination test may also be the sign of possible impulsivity manifested as increased "interest" to the rewarded new task.

Increased phospho-tau/tau ratio was reported in many studies (47,69,75,81,86,89,91,92,99,102,105,127) and was found in young Long-Evans rats, too (66). In the current study we detected a marginally significant (62%) increase in the young naïve and a significant (95%) increase in the old experienced animals, while in young trained rats a non-significant 68% increase was observed. Apart from the statistical significance, a moderate increase was found in all of the examined treated groups compared to the control animals.

Elevated β -amyloid level is a common finding in the literature (47,84,86,89,93,99,102,104–106), also in Long-Evans rats (66), however in our study, it was only confirmed in young naïve rats. (*N.B.:* in the few studies that examined old animals (57–59) β-amyloid accumulation was not measured.) Taking into consideration that in the literature, 4-6 months old animals were most commonly used, a possible explanation for this seemingly contradictory result, is that our 12 and 25 months old rats already had high β -amyloid level, which entailed a possible ceiling effect. Age-related increase in amyloid level in rats has already been described in the literature (131,132). Our finding of a significant age-dependent increase in β-amyloid level in our control animals supports this assumption. The rats at the age of 13 months showed appr. 3-fold higher level of β -amyloid than the rats at the age of 5 months, while STZ could only cause a 2.2-fold increase in β-amyloid level in 5-month-old unexperienced animals. Since our 13-and 27-months old animals also

produced cognitive impairment, the above results also suggest that the cognition-impairing effect of STZ was not necessarily related to β-amyloid formation.

Impulsive-like behavior has not been described in the literature yet, and this finding may suggest a new direction of research in the future. Impulsivity is not among the non-cognitive symptoms of AD (133,134), rather, impulsivity and disinhibition are well known symptoms of frontotemporal dementia (FTD) (134–136). FTD lacks amyloid pathology and characterized by increased phospho-tau/total tau biomarker (137,138). Although AD and FTD share some common pathological mechanisms (increased phospho-tau/tau) and symptoms (memory loss) they are etiologically different. For example, altered insulin signaling was also described in patients with FTD, however in opposite direction than that in AD: insulin and IR expressions were elevated in the frontal lobe, IGF-1 receptor was upregulated in the frontal and temporal lobes (139). In the light of this difference consider the opposite changes in the IDE level of STZ-treated Wistar (69,87) and Long-Evans rats (22,67).

6. Conclusion

The main conclusion of the study with young unexperienced Long-Evans rats was that STZ differently altered the Long-Evans strain compared to the albino strains. Long-Evans rats are likely less sensitive to STZ treatment but 3 × 1.5 mg/kg dose of STZ was sufficient to induce behavioral and biochemical changes. Differences between rat strains in the effect of STZ has already been demonstrated . Bloch et al. (45) found obesity and peripheral metabolic abnormalities in icv. STZ treated Lewis rats what is not observed in Wistar strain. Fine et al. (67) and Delikkaya et al. (22) found increased IDE in STZ-treated Long-Evans rats while in the Wistar literature opposite changes are reported. 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 amyloid-β mediated. 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 amyloid-β) hints at a possible FTD connection.

How good model of AD is the icv. STZ method then?

The obtained results and our literature survey suggest that the icv. STZ is not a superior model of AD and far from the claim of De la Monte et al. (66) “The intracerebral streptozotocin (i.c. STZ) model replicates the full range of abnormalities in sporadic AD”. It has certain promising features (detailed in the Introduction) 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. A limitation of the icv. STZ model is the lack of NFTs and amyloid plaques. Even in case of the frequently detectable symptoms, contradictory results were reported in several articles (see in the Discussion section). It is of concern, that in most of the articles just one or two behavioral tests (typically MWM and/or PAL) are used, which does not give a comprehensive picture of cognitive deterioration. Furthermore, there is a diversity in the timing of behavioral tests, too.

Based on the literature data no clear-cut dose-dependence can be established. In addition to strain differences, sex differences were also observed in the sensitivity of icv. STZ treatment. Bao et al. (48) observed cognitive impairment and elevated phospho-tau and β -amyloid level in male but not in female Sprague-Dawley rats. In another study, Biasibetti et al. (49) found that female Wistar rats were more resistant to the icv. STZ induced alterations.

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 on 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 currently have certain limitations and needs further elaboration.

Required to examine the effect of the STZ on experienced old female rats because there is a sex difference in STZ treated rats and AD patients. It would be necessary to expand the tested cognitive domains (e.g. for working memory) and to examine the development of insulin resistance (measuring insulin, IR, IGF, IGF-R or IDE levels). Further behavioral and biochemical experiments should be conducted to investigate the relationship between the STZ model and FTD as well. Given the obvious strain difference in response to icv. STZ, experienced old Wistar rats should also be involved in the experiments. 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. Although these proposed studies are resource-intensive and do not allow fast publication, they represent the most appropriate way to strengthen and specify the translational validity of the icv. STZ model.

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7. Summary

The intracerebroventricularly (icv.) injected streptozotocin (STZ) induced brain state is a widely used model of sporadic Alzheimer-disease (AD) producing many symptoms of the human disease (cognitive decline, increase in β-amyloid and phospho-tau level). However, the model has predominantly been used with young, naive albino rats in the literature. We postulate that the translationally most relevant animal population of an AD model should be that of aged rats with substantial learning history. The objective of the doctoral work was to integrate the icv. STZ model in our complex cognitive test battery where we use this strain because of its superior cognitive capabilities. We implemented the model in several steps. In the first step, we transferred the model to young, naïve Long-Evans rats by performing two experiments (EXP1, EXP2). At EXP1, rats were treated with 2x1.5 mg/kg icv. STZ (the most frequently used dose in the literature). Since this treatment was ineffective, at EXP2 animals were treated with 3x1.5 mg/kg icv. STZ. We found significant impairment in the novel object recognition (NOR) test (recognition memory) and elevated β-amyloid level in the STZ treated group in addition to slower learning of the five-choice serial reaction time test (5-CSRTT, attention) and a trend for increased phospho-tau/tau ratio. In the Morris water-maze (MWM, spatial learning) and passive avoidance learning (PAL, fear memory) no effect of STZ was observed. In the second and third step, Long-Evans rats of 10 and 23 months age with acquired knowledge in 5-CSRTT, a cooperation task (social memory), MWM and "pot-jumping" exercise (procedural learning) were treated with 3 × 1.5 mg/kg icv. STZ and their performance were followed for 3 months in the above and additional behavioral assays. Both STZ-treated age groups showed significant impairment in the MWM and novel object recognition test but not in passive avoidance and fear conditioning paradigms. In young STZ treated rats, significant differences were also found in the 5-CSRTT and pot jumping test while in old rats a significant increase in hippocampal phospho-tau/tau protein ratio was observed. No significant difference was found in the cooperation and pairwise discrimination (visual memory) assays and hippocampal β-amyloid levels. STZ treated old animals showed impulsivity-like behavior in several tests. Our findings suggest that Long-Evans rats may be less sensitive to the STZ treatment than Wistar rats highlighting the importance of strain diversity in modelling human diseases. In experienced rats the examined cognitive domains showed different sensitivity to STZ. The observed cognitive and non-cognitive activity pattern in aged experienced rats call for more extensive studies with the icv. STZ model to further strengthen and specify its translational validity.

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9. 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.
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Own publications not involved in the current thesis

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Intracerebroventricularly Injected Streptozotocin Exerts Subtle Effects on the Cognitive Performance of Long-Evans Rats

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Intracerebroventricularly injected streptozotocin (STZ)-induced learning impairment has been an increasingly used rat model of Alzheimer disease. The evoked pathological changes involve many symptoms of the human disease (cognitive decline, increase in β-amyloid and phospho-tau level, amyloid plaque-like deposits). However, the model has predominantly been used with Wistar rats in the literature. The objective of the current study was to transfer it to Long-Evans rats with the ulterior aim to integrate it in a complex cognitive test battery where we use this strain because of its superior cognitive capabilities. We performed two experiments (EXP1, EXP2) with three months old male animals. At EXP1, rats were treated with 2 × 1.5 mg/kg STZ (based on the literature) or citrate buffer vehicle injected bilaterally into the lateral ventricles on days 1 and 3. At EXP2 animals were treated with 3 × 1.5 mg/kg STZ or citrate buffer vehicle injected in the same way as in EXP1 at days 1, 3, and 5. Learning and memory capabilities of the rats were then tested in the following paradigms: five choice serial reaction time test (daily training, started from week 2 or 8 post surgery in Exp1 or Exp2, respectively, and lasting until the end of the experiment); novel object recognition (NOR) test (at week 8 or 14), passive avoidance (at week 11 or 6) and Morris water-maze (at week 14 or 6). 15 or 14 weeks after the STZ treatment animals were sacrificed and brain phospho-tau/tau protein ratio and β -amyloid level were determined by western blot technique. In EXP1 we could not find any significant difference between the treated and the control groups in any of the assays. In EXP2 we found significant impairment in the NOR test and elevated β-amyloid level in the STZ treated group in addition to slower learning of the five-choice paradigm and a trend for increased phospho-tau/tau ratio. Altogether our findings suggest that the Long-Evans strain may be less sensitive to the STZ treatment than the Wistar rats and higher doses may be needed to trigger pathological changes in these animals. The results also highlight the importance of strain diversity in modelling human diseases.

Keywords: Alzheimer disease model, STZ icv., cognitive test battery, learning impairment, β-amyloid, phospho-tau

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INTRODUCTION

The bitter experience of anti-dementia drug development over the past 15 years has been that clinical trials of potential cognitive enhancers have resulted in 100% failure, mostly due to lack of efficacy (Cummings et al., 2014). One of the main reasons for the serial failures is the low translational value of animal experimental models predicting human efficacy. In the case of Alzheimer's disease (AD) therapeutic approaches were based almost exclusively on the amyloid cascade hypothesis (Barage and Sonawane, 2015), and its key models were transgenic mouse lines carrying human mutant transgenes characteristic for the familial form of the disease. These strains are characterized by massive human β-amyloid overproduction, but this can be considered a model of amyloid intoxication rather than the disease itself, as they did not show tau pathology and the observed cognitive defects did not correlate with histological changes (Foley et al., 2015). The series of failures in clinical trials (Schneider et al., 2014) have raised serious doubts not only about the validity of the transgenic models but also about the validity of the amyloid theory itself (Herrup, 2015). For these reasons, non-transgenic models of sporadic AD have again become the focus of research. One prominent representative of these is the intracerebroventricularly (icv.) injected streptozotocin (STZ)-induced insulin-resistant brain state (Chen et al., 2013; Salkovic-Petrisic et al., 2013). The theoretical basis of the model is the cerebral insulin resistance in AD, which is why the disease is also referred to as type 3 diabetes (Chen and Zhong, 2013). As a result of insulin resistance induced by STZ treatment (Craft, 2006; Agrawal et al., 2011; De Felice et al., 2014), AD-like pathology develops (increased phospho-tau at 1 month post-injection, β -amyloid at 3 months, appearance of plaques at 6 months) associated with cognitive deficits (already at 1 month) (Knezovic et al., 2015). Based on the data to date, it appears to be a more adequate model than transgenic mice (Salkovic-Petrisic et al., 2013) and has the additional advantage of being applicable to rats.

Our group elaborated and established a rat cognitive test battery and testing protocol for more reliable prediction of clinical efficacy of putative cognitive enhancer drugs (Gyertyán, 2017; Gyertyán et al., 2020). According to the protocol, several cognitive tasks representing different cognitive domains were taught to the same cohort of Long-Evans rats, for example, five-choice serial reaction time task (5-CSRTT) for attention, a cooperation task for social cognition (Kozma et al., 2019), Morris water maze paradigm for spatial memory, "pot-jumping" exercise for procedural memory (Ernyey et al., 2019). Hereby we created a population with "widespread knowledge" (Gyertyán et al., 2016). The Long-Evans strain was chosen for its good learning capability, which is an essential requirement in a system imposing heavy cognitive load on the subjects. The effect of a particular impairment method on the various cognitive functions could then be simultaneously measured in this trained population. These impaired states served then as the target of potential cognitive enhancer treatments in a "clinical trial-like", vehicle controlled, double blind, randomized experimental design (Gyertyán et al., 2020). The icv. STZ-model could be integrated into this testing protocol as a distinguished, particularly useful impairing method of high translational potential. As the model has been used with Wistar–and to a lesser extent Sprague-Dawley rats in the literature, transferring it into Long-Evans animals is the first step toward this integration. The objective of the current study was to try to reproduce the cognitive and biochemical changes described in Wistar rats in the literature in naive Long-Evans rats as well.

METHODS AND MATERIALS

Animals

Eight-nine weeks old male Long-Evans rats (Janvier Labs, Le Genest-Saint-Isle, France) were used in this study; 18 subjects weighing 240–280 g in experiment 1 (EXP1), and 24 subjects (210–270 g) in experiment 2 (EXP2). Animals were kept three per cage (1376 cm^2 polycarbonate cages with paper tubes and wooden bricks as environmental enrichment tools) under reverse light dark cycle (dark phase from 4 am to 4 pm). Food (commercial pellet rat feed R/M–Z + H produced by SSniff Spezialdiäten GmbH, Soest, Germany) was available *ad libitum* up to the end of the post-injection recovery period; after that the animals had a restricted food access: the amount of the food was 45 g for three rats and it was supplied before the end of the dark phase. Drinking water was available ad libitum over the whole course of the experiment. The animals were intensively handled before and during the experiments. At the end of the behavioral measurements, they were anaesthetized by isoflurane and decapitated to remove their hippocampus for the western blot measurements. The experiments were authorized by the regional animal health authority in Hungary (resolution number PE/EA/785–5/2019) and conformed to the Hungarian welfare law and the EU 63/2010 Directive.

Intracerebroventricular Streptozotocin Treatment

During EXP1, 3 mg/kg icv STZ (Sigma-Aldrich, St. Louis, MO, United States) divided into two 1.5 mg/kg doses were given bilaterally at day 1 and day 3. A volume of 2 μL/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. At EXP2, rats were treated with 4.5 mg/kg STZ split into three equal doses administered on day 1, 3, and 5 (**Figure 1**). In both experiments, STZ was dissolved in 0.05 M citrate buffer pH 4.5 [sodium citrate dihydrate (0,0228 M) and citric acid (0,0272 M), Santa Cruz Biotechnology (Santa Cruz, CA, United States)]. The control groups received vehicle treatment in both experiments.

In EXP1, rats were anesthetized by sodium pentobarbital (60 mg/kg, i.p.) at both injections. Unfortunately, one animal from the control group could not recover from anesthesia. In EXP2, rats received anaesthesia via a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg ip.) during the first drug administration and isoflurane (4% in pure oxygen) during the

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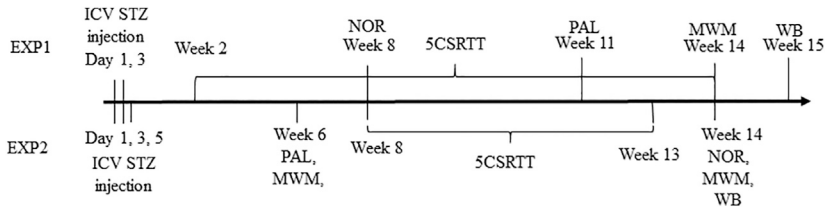

FIGURE 1 | Timeline of the experiments (EXP, experiment; ICV, intracelebroventricular; STZ, streptozotocin; NOR, novel object recognition; 5CSRTT, five choice reaction time task; PAL, passive avoidance learning; MWM, Morris water maze; WB, western blot).

2nd and 3rd surgeries. Animals were placed in a stereotactic apparatus (Stoelting, Wood Dale, IL, United States) and laid on a heating bench (37°C) (Supertech Instruments, Pécs, Hungary). Midline incision on the skin was made and the surface of the skull was cleaned. Drilled holes at the place of the injection was made by dental drill. The ICV coordinates were: 0.72 mm posterior to Bregma, 1.5 mm lateral to sagittal suture, 3.6 mm ventral of the surface of the brain (Noble et al., 1967). A guide cannula was placed into the drilled hole in the skull and STZ was infused by a Hamilton syringe via a microinjection pump (CMA/100, CMA/Microdialysis Ab, Stockholm, Sweden); the injection speed was 5 min/hole. The needle was left in place for an additional 2 minutes then the guide canule was removed and the wound sutured. After the last treatment, the holes were closed by bone-cement. After the surgery, rats were given buprenorphine (0.05 mg/kg i.p.) and lidocaine was applied to the wound as analgesics. During the period of the surgeries and one week thereafter the animals had *ad libitum* food access. Until the wounds healed (approximately two weeks), the animals were kept separately.

Behavioural Assays

Novel Object Recognition

The test apparatus was a 48x48x42 cm box with bedding material on the bottom where the behaviour of the animals were recorded by a video camera system. Before the testing day, rats were habituated to the test box for 3 minutes (EXP1) or 10 minutes (EXP2). 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 box. The objects were placed 10 cm from the diagonally opposite corners and 40 cm from each other. After a delay of 80 minutes (EXP1) or 60 minutes (EXP2), in the retention trial one of the objects was changed to a novel one and the animals had 3 minutes again to explore them. The recognizable objects were a glass jar and a plastic jar in EXP1 and a plastic bottle filled with gravel and a glass bottle filled with blue dye solution in EXP2. Exploration time of each object was the registered parameter. Recognition memory was characterized by the discrimination index according to the following equation: $DI = \frac{new\ object - old\ object}{new\ object + old\ object} \times 100$. Animals which explored the objects for less than 10 seconds or explored only one of the two objects in any of the trials were excluded from the experiment (2 animals from the control group and one from the STZ group in EXP1, and one animal from the control group and two rats from the STZ group in EXP2).

Passive Avoidance Learning

The type of the experiment was a step through passive avoidance test. The apparatus consisted of a light and a dark chamber separated by a guillotine door. The test consisted of two parts, the acquisition trial and 24 hours later the retention trial. During the trials the rats were placed into the light chamber and 30 sec later the door opened and the animal could cross into the dark chamber. In the acquisition trial the animals had 180 sec (cut off time) to enter the dark compartment of the device, whereas at the retention trial the cut off time was 300 sec. When the rat passed through to the dark side, the door closed and after a 3 seconds delay a mild foot shock (0,6 mA, 3 sec) was delivered. The animal was left in the dark compartment for an additional 5 seconds after the shock. The measured parameters were entry latencies into the dark compartment in the acquisition and the retention trials.

Morris Water Maze

The apparatus was a black circular pool (diameter 190 cm, depth 60 cm) filled with water (38 cm, 23 ± 1°C) and containing a non-visible round escape platform (10 cm diameter) placed 0.5 cm below the water surface. The platform was located in the south-east (SE) quadrant, 40 cm from the edge of the pool. On the wall of the experimental room extra-maze cues were placed to facilitate the orientation during swimming. At the start of a trial the rat was placed into the pool at one of the four possible start points (North, East, West or South rotated in a systemic manner) had 3 minutes to find the hidden escape platform. When the animal didn't find it, it was gently guided to the platform. Rats were allowed to spend 30 sec on the platform then were taken out, dried with a cloth and replaced in their home-cage. During the acquisition phase the animals were trained in 3 daily trials for two (EXP1) or three (EXP2) consecutive days. The interval between the trials was 30 min. Escape latency was measured and swimming path was recorded by Smart v3.0 video tracking system software (Panlab, Barcelona, Spain). Two days after the last acquisition trial, the animals were tested in a probe trial when the hidden platform was removed from the maze. In this measurement, the rats had 2 minutes to explore the maze, the measured parameter was the time they spent in the target quadrant (where the platform had been located during the acquisition trials). After a 30 min delay, the hidden platform was replaced to the maze at a different position [north-west (NW)], and 3 more acquisition trials were run. With the EXP2 group, 3 months after the STZ treatment an acquisition

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session (hidden platform located at NW) and two days after a single probe trial was made.

5-Choice Serial Reaction Time Task

5CSRTT device consist of a 31x35x34 cm test box (cat. no. 259920) (TSE Systems, Bad Homburg vor der Höhe, Germany). The boxes were equipped with 5 nose-poke modules on the back wall and with a magazine at the front wall. During the task, rats had to nose-poke into that hole where the light was turned on. After 5 s inter-trial interval, in one randomly selected nose-poke module a 1 sec long stimulus was presented. The animal made a correct response if nose-poked into this hole during the stimulus presentation or within 5 s afterwards (limited hold). Correct responses were rewarded with a pellet delivered into the magazine. Nose-poke into the magazine initiated the next trial. The animal made an incorrect response if nose-poked into one of the holes where the stimulus was not presented. An omission response was recorded when the rat did not make any nose-poke up to the end of the limited hold. Incorrect responses and omissions were followed by 5 s time-out punishment, when the house light was turned off. After the time-out, the house light was set back and the rat could start the next trial by nose-poking into the magazine. The animal made a premature response, if nose-poked into any of the holes during the inter-trial interval. These responses were also punished with time-out. Length of a daily test session was 20 min. Rats were trained for the task in stages with gradually decreased stimulus duration from 30 to 1 s. Animals could step to the next training stage, if they collected at least 40 (EXP1) or 30 rewards (EXP2) during a training session. One animal which did not even reach the 1st stage (learning to use the nosepoke modul) was excluded from the experiment. The outcome parameters were the days needed to complete the final stage and the learning curve plotted as average learning stage in function of training days.

Western Blot

After the behavioral tests, the animals were decapitated, their brain were removed and both hippocampi were dissected then frozen and stored at −80°C. Hippocampal tissues were homogenized with TissueLyser (Qiagen, Venlo, Netherlands) in lysis buffer containing 200 mM NaCl, 5 mM EDTA, 10 mM Tris, 10% glycerine, and 1 g/ml leupeptin (pH 7.4), supplemented with a protease inhibitor cocktail (cOmplete ULTRA Tablets, Roche, Basel, Switzerland) and PMSF (Sigma, St. Louis, MO, United States). The homogenized lysates were centrifuged twice at 1,500x g and 4°C for 15 min, then the supernatants were collected and their protein concentration was measured by the bicinchoninic acid assay (Thermo Fisher Scientific, Waltham, MA, United States). Equal amount of protein (20 µg) was mixed with Pierce Lane Marker reducing sample buffer (Thermo Fisher Scientific, Waltham, MA, United States), and loaded and separated in a 4–20% precast Tris-glycine SDS polyacrilamide gel (Bio-Rad, Hercules, CA, United States). Proteins were transferred electrophoretically onto a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, United States) at 200 mA overnight. Membranes were blocked with 5% nonfat dry milk (Cell Signaling Technology, Leiden, Netherlands) in Tris buffered saline containing 0.05% Tween-20 (0.05% TBS-T; Sigma, St. Louis, MO, United States) at room temperature for 2 h. Membranes were incubated with primary antibodies against PHF1 (sc515013, 1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA, UnitedStates), Tau (sc32274, 1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA, UnitedStates) and β-Amyloid (sc28365, 1:500, Santa Cruz Biotechnology, Santa Cruz, CA, UnitedStates) 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

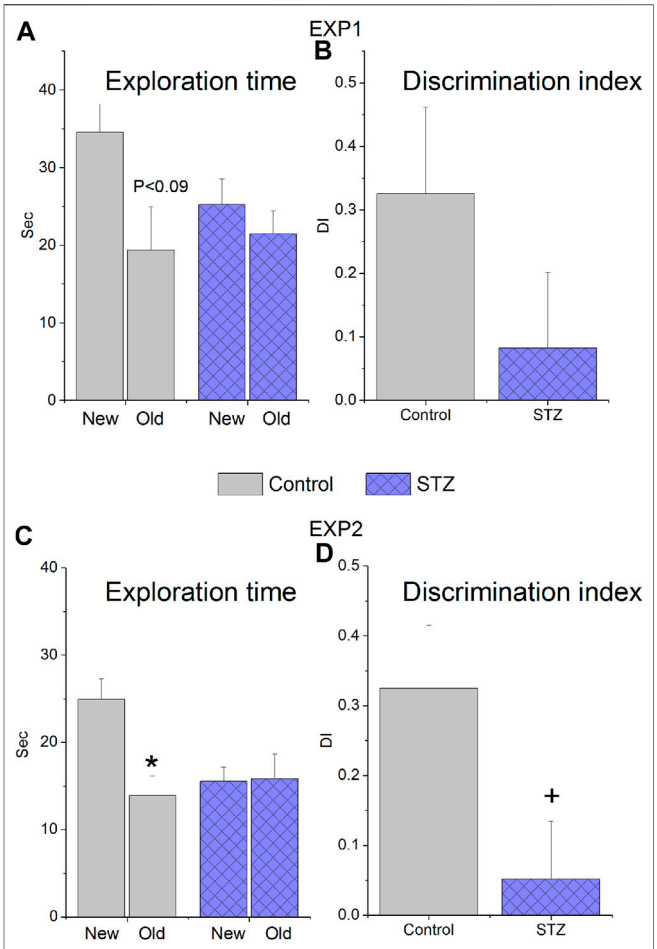

FIGURE 2 | Novel Object Recognition performance of rats treated with icv. streptozotocin (STZ) or citrate buffer (control). Means ± SEM values are shown. **(A,B)** Results of experiment 1 (EXP1), when the animals received 2 × 1.5 mg/kg icv. STZ 8 weeks before the test. Exploration time of the familiar (old) and unfamiliar (new) objects marginally significantly differ in the control group (paired *t*-test: $t(5) = 2.10$, $p = 0.089$) but not in the STZ group (paired *t*-test: $t(7) = 0.70$, ns) and the calculated discrimination indices (DI) did not significantly differ (unpaired *t*-test: $t(12) = 1.33$, ns) **(C,D)** Results of EXP2, when the animals received 3 × 1.5 mg/kg icv. STZ 14 weeks before the test. The control group explored the new object for significantly longer time than the old one (paired *t*-test: $t(10) = 3.53$, $p < 0.005$) while the STZ-treated rats spent equal time in examining the objects (paired *t*-test: $t(9) = -0.10$, ns). The discrimination indices (DI) of the two groups were also significantly different (unpaired *t*-test: $t(19) = 2.21$, $p < 0,05$) *$p < 0.05$ vs "new", + < $p < 0.005$ vs "control".

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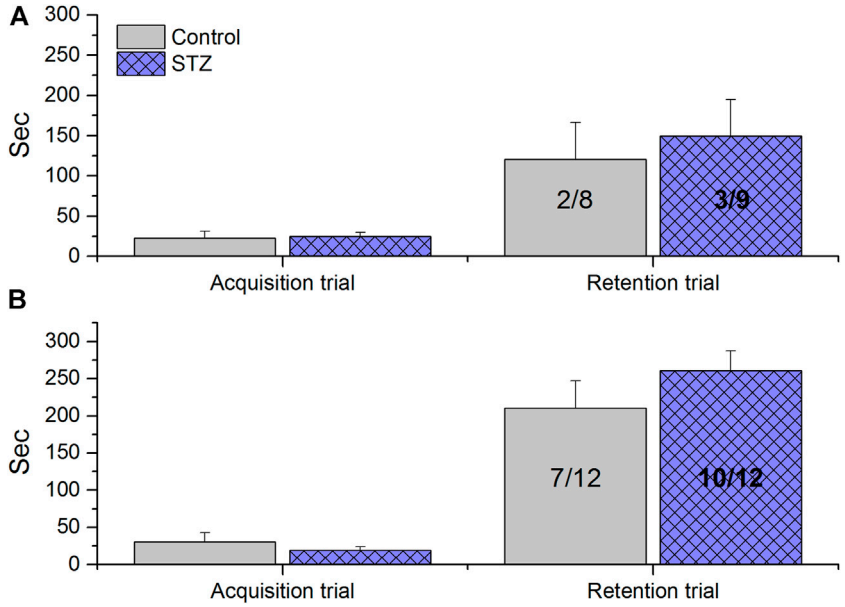

FIGURE 3 | Passive Avoidance Learning results of rats treated with icv. streptozotocin (STZ) or citrate buffer (control). Columns show means ± SEM values of entry latencies, numbers inside the columns indicate the not entered/total number of animals. **(A)** Results of experiment 1 (EXP1), when the animals received 2 × 1.5 mg/kg icv. STZ 11 weeks before the test. No significant difference was observed between control and STZ-treated animals (unpairedt-test acquisition trial: $t(15) = -0.23$, ns; unpaired *t*-test retention trial: $t(15) = -0.45$, ns; Fischer exact p, two tailed test $p = 1.0$, ns). **(B)** Results of experiment 2 (EXP2), when the animals received 3 × 1.5 mg/kg icv. STZ 6 weeks before the test. No significant difference was observed between control and STZ-treated animals (unpaired *t*-test acquisition trial: $t(22) = 0.85$, ns; unpaired *t*-test retention trial: $t(22) = -1.10$, ns; Fischer exact two tailed test $p = 3707$).

of the β -Amyloid. Signals were detected with a chemiluminescence kit (Bio-Rad, Hercules, CA, UnitedStates) by Chemidoc XRS+ (Bio-Rad, Hercules, CA, UnitedStates). The intensity of the samples was measured by Image Lab software (version 4.1, Bio-Rad, Hercules, CA, UnitedStates). Phospho-specific antibody was removed with Restore™ Western Blot Stripping Buffer (Thermo Fisher Scientific, Waltham, MA, UnitedStates) before the incubation of the corresponding total protein antibody.

Statistics

Group means ± standard error were calculated and significance was determined by unpaired *t*-test (5CSRTT days to complete, NOR discrimination index, PAL, MWM probe trial, WB), paired *t*-test (NOR discrimination index), Fischer exact test (PAL frequency) or repeated measures ANOVA (MWM escape latencies) using the Statistica 13.5.0.17 software package (TIBCO Software Inc.). The sigmoidal fits to the 5CSRTT learning curves were performed by the Origin 2015 software (OriginLab Corporation). In addition, a multivariate ANOVA was performed on the following variables in both experiments: NOR discrimination index, days needed to reach the final stage in the 5-CSRTT, phospho-tau/tau ratio, β-amyloid level (Statistica 13.5.0.17).

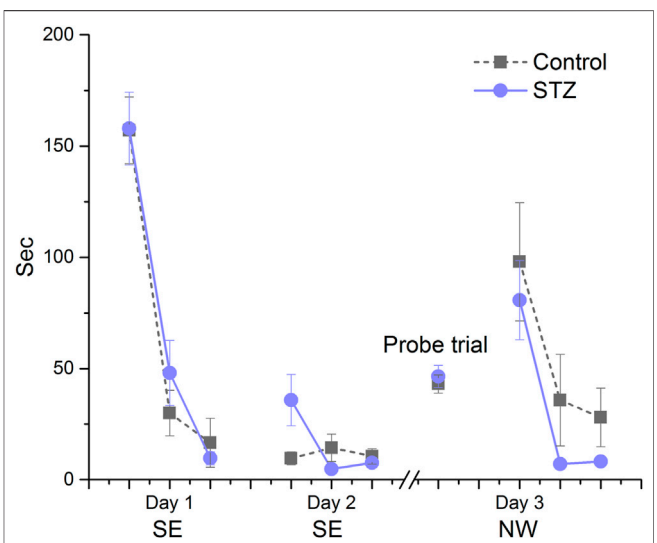

FIGURE 4 | Learning performance in the Morris water-maze in EXP1. Means ± SEM of escape latency values are shown except in the probe trial where the time spent in the target quadrant is depicted. SE and NW indicate the position of the escape platform. There was no significant difference between groups in the acquisition trials on days 1 and 2 (group effect: $F_{(1,15)} = 0.89$, ns; Day × trial × treatment interaction: $F_{(2,30)} = 1.94$, ns), in the probe trial (unpaired *t*-test: $t(15) = -0.51$, ns) and during re-acqusition on Day 3 (group effect: $F_{(1,15)} = 1.60$, ns, Day × treatment interaction: $F_{(2,30)} = 0.11$, ns).

RESULTS

Novel Object Recognition

In EXP1, STZ-treated animals explored less the unfamiliar new object than the control rats (**Figure 2A**) and their DI value was also much lower (0.33 and 0.08 in the control and STZ group, respectively, **Figure 2B**), nevertheless, due to the low number of

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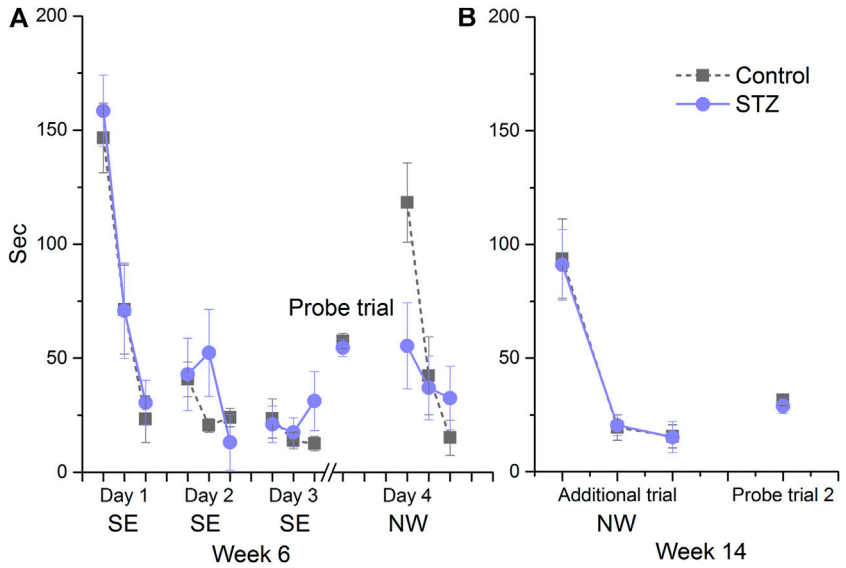

FIGURE 5 | Learning performance in the Morris water-maze in EXP2. Means ± SEM of escape latency values are shown except in the probe trial where the time spent in the target quadrant is depicted. SE and NW indicate the position of the escape platform. **(A)** There was no significant difference between groups in the acquisition trials on days 1, 2 and 3 (Day x trial × treatment interaction: $F_{(4,88)} = 1.80$, ns) in the probe trial (unpaired *t*-test: $t(22) = 0.58$, ns)) and during the re-acquisition at day 4 (Day × treatment interaction: $F_{(2,44)} = 3.79$, ns). **(B)** No significant difference was detected during the additional acquisition session (Day × treatment interaction: $F_{(2,44)} = 0.01$, ns) and the second probe trial (unpaired *t*-test: $t(22) = 0.66$, ns).

animals remained in the experiment (n = 6 and n = 8 for control and STZ, respectively) the difference was not statistically significant (**Figure 2B**). In EXP2, control animals spent significantly more time in examining the unfamiliar object (24.9 s) than the old one (13.9 s) whereas STZ-treated rats equally explored both (15.6 s and 15.8 s for new and old, respectively) (**Figure 2C**). The DI values of the two groups (0.32 and 0.05 for control and STZ, respectively, **Figure 2D**) were significantly different.

Passive Avoidance Learning

There was no significant difference between the learning performances of groups either in acquisition or retention trials in any of the experiments (**Figures 3A,B**).

Morris Water-Mate

In EXP1 this assay was carried out at week 14. Control and treated animals similarly performed in the acquisition trials (days 1–2, **Figure 4**). All of the rats successfully learned the location of the hidden platform with similar decrease in their escape latency. The animals spent the same amount of time in the target quadrant during the probe trial, furthermore no significant difference was found between the groups during the re-acquisition trials when the platform was replaced to a new location (**Figure 4**). In EXP2, MWM performance was first measured at week 6 (**Figure 5A**). Again, no significant difference was detected in the performance of the control and STZ-treated groups in the three phases of the test. To examine the possible later development of cognitive impairment, an additional acquisition session and probe trial were carried out at week 14; nonetheless there was no significant difference between the groups (**Figure 5B**).

5-Choice Serial Reaction Time Task

In EXP1, STZ-treated and control animals showed intersecting and overlapping learning curves (inflection points of the fitted sigmoid regression curves were 24.7 and 25.8 days, respectively) and the days needed to reach the maximum learning stage were the same (43.8 and 43.8 days, respectively) (**Figure 6**). In EXP2, however, the control group learned significantly faster shown by the two days difference in the midpoint of the fitted sigmoid regression curves (11.4 and 13.4 days in the control and STZ-treated group, respectively, **Figure 7**), furthermore, the STZ-treated animals needed 3 days more to complete the task (21.2 *vs* 18.0 days in the control group), though this difference was not significant.

Western Blot Measurements

In EXP1, Western blot analysis revealed no significant difference in phospho-Tau/Tau ratio and β-amyloid level between vehicle- and STZ-treated animals (**Figures 8A,C**). In EXP2 we found a marginally significant elevated phospho-tau/tau ratio (**Figure 8B** significant increase in the β -amyloid level in the STZ-treated animals (**Figure 8D**).

Multivariate Analysis of Variance

We found more pronounced effects in four out of the six assays in EXP2 vs EXP1, although in themselves they were not always statistically significant. To statistically analyze the overall difference between the two experimental protocols we performed a multivariate ANOVA on four variables each from one of these 4 assays: phospho-tau/tau ratio, β-amyloid level, NOR discrimination index, and days needed to reach the final stage in the 5-CSRTT. The difference between the control and

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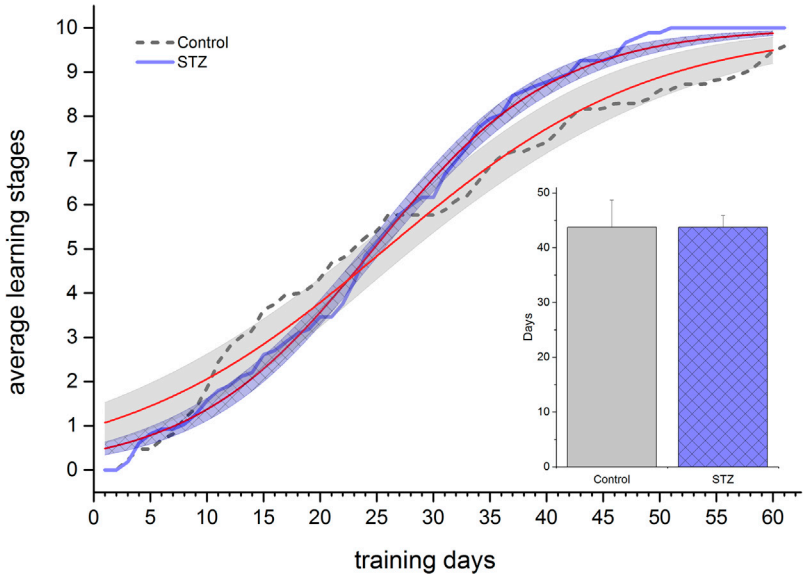

FIGURE 6 | Learning performance in the 5 Choice Reaction Time task in EXP1. Learning curves of control and STZ-treated animals are depicted. Shaded areas show the 95% confidence band of the fitted sigmoidal regression curves (thin [red] lines). The column chart inset shows the number of days taken to reach the maximum stage. Means ± SEM values are shown. No significant difference was detected either between the learning curves or in the days elapsed until reaching the final stage (unpaired *t*-test: $t(15) = 0.25$, ns).

STZ groups was significant in EXP2 (Wilks λ = 0,391, F(4,13) = 5,054; p = 0,011) whereas it was not significant in EXP1 (Wilks λ = 0,750, F(4,7) = 0,583; p = 0,685298344).

DISCUSSION

The icv. STZ-induced brain pathology has been an increasingly used model of Alzheimer's disease. The preferred subjects of the model are Wistar and to a lesser extent–the Sprague-Dawley rats and mice. A few papers were published on Lewis rats (Blokland and Jolles, 1993; Bloch et al., 2017) but pigmented rats–up to our knowledge have not been examined in the model yet. However, apart from the species and strain, several variations of other parameters of the model have been published which offered various options to choose while transferring the model to the Long-Evans strain.

1. Dosing of STZ: the most common dosing is 3 mg/kg split into two 1.5 mg/kg doses injected with two days difference (Sonkusare et al., 2005; Pathan et al., 2006; Prakash and Kumar, 2009; Salkovic-Petrisic et al., 2011, Salkovic-Petrisic et al., 2015; Hashemi-Firouzi et al., 2018; Knezovic et al., 2018; Majkutewicz et al., 2018; Yamini et al., 2018) but single 1 mg/kg (Salkovic-Petrisic et al., 2006, 2015; Grünblatt et al., 2007), 1.5 mg/kg (Blokland and Jolles, 1993; Jee et al., 2008; Deng et al., 2009), 2 mg/kg (Moreira-Silva et al., 2018) and 3 mg/kg (Rodrigues et al., 2010; Correia et al., 2013; Osmanovic Barilar et al., 2015; Samy et al., 2016; Zappa Villar et al., 2018; Bavarsad et al., 2020) doses are also applied. We chose the first regimen.

2. Follow-up period after the injection: the majority of the published studies applied a one month long post-injection period

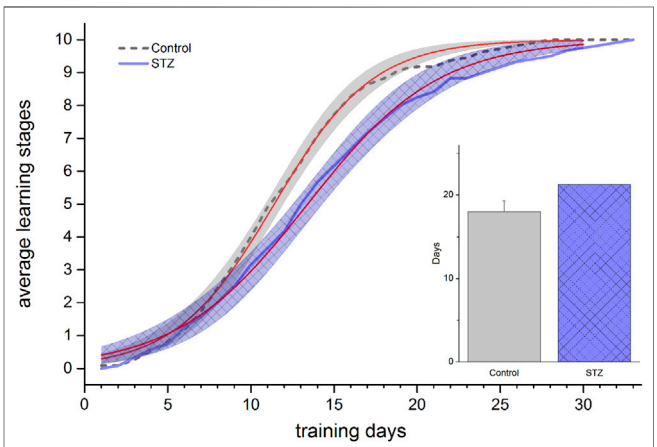

FIGURE 7 | Learning performance in the 5 Choice Serial Reaction Time task in EXP2. Learning curves of control and STZ-treated animals are depicted. Shaded areas show the 95% confidence band of the fitted sigmoidal regression curves (thin [red] lines). The regression lines significantly differ (no overlap between the confidence bands). The column chart inset shows the number of days taken to reach the final stage. Means ± SEM values are shown. No significant difference was detected between the two groups (unpaired *t*-test: $t(21) = -1.46$, ns).

(Li et al., 2012; Correia et al., 2013; Zhou et al., 2013; Hashemi-Firouzi et al., 2018; Zappa Villar et al., 2018) or even shorter, 2–3 weeks intervals (Blokland and Jolles, 1993; Sonkusare et al., 2005; Jee et al., 2008; Deng et al., 2009; Yamini et al., 2018). There are some studies where longer, 3 months follow-up periods were used (Salkovic-Petrisic et al., 2006, Salkovic-Petrisic et al., 2011, Salkovic-Petrisic et al., 2015; Knezovic et al., 2015; Samy et al., 2016;

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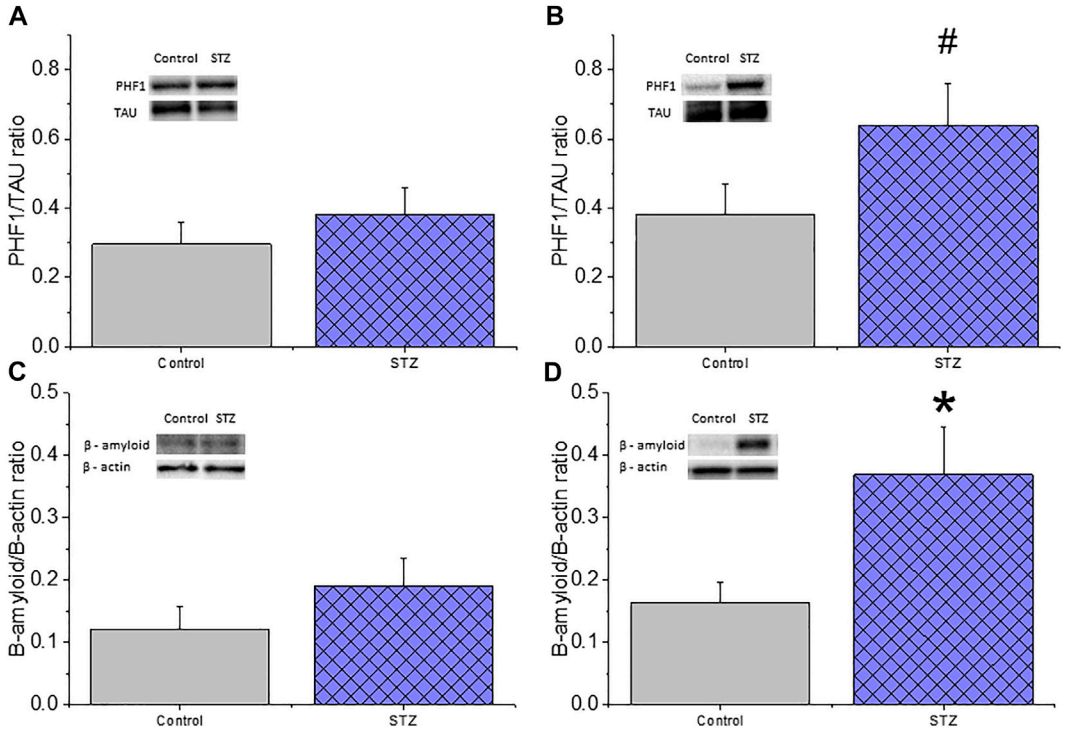

FIGURE 8 | The effect of icv. STZ or citrate buffer (control) treatment on the tissue protein levels of phospho-Tau (A and B) and β-amyloid (C and D) in EXP1 (A and C) and EXP2 (B and D) measured by Western blot. Means ± SEM values are shown. There was no significant difference between the groups in phospho-tau/tau ratio in EXP1 (unpaired *t*-test: $t(13) = -0.85$, ns) **(A)** whereas a trend for an increase in the STZ group can be seen in EXP2 (unpaired *t*-test: $t(21) = -1.74$, $p = 0.10$) **(B)**. Also, there was no significant difference in β-amyloid level in EXP1 (unpaired *t*-test: $t(13) = -1.13$, ns) **(C)** while significantly elevated β-amyloid level was found in the STZ-treated group in EXP2 (unpaired *t*-test: $t(20) = -2.45$, $p < 0.05$ **(D)** $^{*}<0.05$, $^{\#}<0.10$.

Knezovic et al., 2018; Ilieva et al., 2019; Voronkov et al., 2019) In two very much informative longitudinal studies (Knezovic et al., 2015; Osmanovic Barilar et al., 2015) changes/impairments were followed up to 9 months. In our study we chose a 3–3.5 months follow up period considering that the method is intended to be a model of Alzheimer's disease, which would imply slowly evolving long term pathological changes and that it allowed to conduct learning tasks requiring several weeks training, like 5-CSRTT.

3. Selected cognitive assays and their timing: PAL (fear memory) and MWM (spatial learning and memory) are by far the most common tasks in the literature with usually significant impairments in the STZ-treated groups. Their timing varies but impairments were shown already at 2–3 weeks post-injection both in the PAL (Blokland and Jolles, 1993; Lannert and Hoyer, 1998; Sharma and Gupta, 2001; Sonkusare et al., 2005; Pathan et al., 2006; Jee et al., 2008; Knezovic et al., 2015; Samy et al., 2016) and in the MWM assays (Pathan et al., 2006; Grünblatt et al., 2007; Prakash and Kumar, 2009; Agrawal et al., 2011; Zhou et al., 2013; Salkovic-Petrisic et al., 2015; Samy et al., 2016; Rajasekar et al., 2017; Majkutewicz et al., 2018; Yamini et al., 2018). Later measurements (1–3 months) also showed impaired performance; PAL: (Samy et al., 2016; Lu et al., 2017; Hashemi-Firouzi et al., 2018; Knezovic et al., 2018; Bavarsad et al., 2020), MWM: (Shoham et al., 2003; Salkovic-Petrisic et al., 2006; Grünblatt et al., 2007; Rodrigues et al., 2010; Li et al., 2012; Correia et al., 2013; Salkovic-Petrisic et al., 2015; Samy et al., 2016; Knezovic et al., 2018; Bavarsad et al., 2020; Liu et al., 2020). Impaired visual recognition memory was also detected in the novel object recognition paradigm 3–8 weeks after STZ-treatment. We chose to apply these cognitive assays. To extend the cognitive domains under investigation we added the 5-CSRTT paradigm (attention). This task requires a long training period therefore we started with it soon after recovery from surgery in EXP1. Timing of the other three assays was based on literature data with taking care to avoid interference with the initial training phase of the 5-CSRTT.

4. Detecting amyloid and tau pathology: increase in phospho-tau/tau ratio was already observed from 2 weeks post-injection and was detected either by Western-blot technique (Salkovic-Petrisic et al., 2006; Grünblatt et al., 2007; Li et al., 2012; Correia et al., 2013; Kosaraju et al., 2013; Zhou et al., 2013; Osmanovic Barilar et al., 2015; Salkovic-Petrisic et al., 2015; Lu et al., 2017; Moreira-Silva et al., 2018; Zappa Villar et al., 2018) or by immunostaining (Lu et al., 2017; Knezovic et al., 2018; Wu et al., 2018). Increase in β-amyloid was shown at later timepoints, about 1.5 months on, by either ELISA (Correia et al., 2013; Kosaraju et al., 2013; Samy et al., 2016; Lu et al., 2017; Wu et al., 2018; Ilieva et al., 2019) or Western-blot (Choi et al., 2014; Kang and Cho, 2014; Zappa Villar et al., 2018) or immunostaining (Salkovic-Petrisic et al., 2006; Choi et al., 2014;

Kang and Cho, 2014; Knezovic et al., 2015; Salkovic-Petrisic et al., 2015). Amyloid-plaque like deposits appeared first at 3 months after STZ injection in the meningeal vessels visualized either by congo red (Salkovic-Petrisic et al., 2006) or by immunostaining (Bloch et al., 2017). At 6 and 9 months they became more pronounced (Salkovic-Petrisic et al., 2011) and progressed into the brain parenchyima (Knezovic et al., 2015). We chose Western blot detection of both phospho-tau and β-amyloid proteins.

During EXP1, we couldn't find any significant difference between the control and STZ-treated groups either in the behavioural assays or in the histological markers β-amyloid and phospho-tau/tau ratio. STZ animals learnt the MWM and 5-CSRTT tasks as well as control animals did. In the PAL test relatively low memory trace could be observed even in the control group. In the NOR assay the control animals showed a sufficient level of discrimination while the STZ-treated rats were much inferior, but due to the low final sample size these differences were not significant.

The results obtained during EXP1 suggested that the dose of STZ may have been inadequate in Long-Evans rats. Therefore, we increased the dose by a factor of 1.5 in EXP2. It was a cautious increase since the exact dose-response relationship is not entirely clear for the icv STZ, and some reports showed dramatic changes even at the 3 mg/kg dose (Bloch et al., 2017). Furthermore, personal communications on unpublished experimental attempts also warned us about severe histological or behavioural toxicity. Instead of increasing the injected dose we added a third 1.5 mg/kg injection partly to avoid acute toxicity, partly to approximate a more prolonged STZ influence.

We also changed the timing of the cognitive assays. We assumed that the early 5 CSRTT learning engagement and the consequential frequent handling of the animals may have had a protective effect against STZ treatment. During EXP2 we dismissed any measurements in the first and a half month to allow a kind of incubation period. PAL and MWM, as the most sensitive tests were the first, while the 5-CSRTT training started afterwards to avoid the above mentioned interference. The NOR test was placed to the end. However, as we did not get any impairment in the MWM, we repeated it at the end of the follow-up period to see if the deterioration could be detectable by then.

During EXP2, we could not again find significant difference in the PAL and MWM tests. In the former, the observed memory trace in the control group was good enough this time to allow to detect an eventual inhibition, yet STZ treated animals performed at least as well as the controls. In the MWM tests, animals showed a similar performance both in the acquisition and probe trials both at the first and the second occasion. These results are in sharp contrast to the findings of the literature referred above, and the discrepancy is not easily explainable. For the MWM one may speculate that STZ treatment may affect visual acuity, which plays an important role in MWM learning, and the superior visual acuity of pigmented rats over white ones (Prusky et al., 2002) may have remained more functional after the STZ-treatment. In most of the cited studies, white STZ-treated rats also showed a learning process but slower than the controls. In the studies of (Prakash and Kumar, 2009; Prakash et al., 2015) STZ-treated animals also found a visible platform significantly slower than the controls. These findings suggest that the impaired MWM performance may resulted from–at least partly–a visual impairment. Certainly, such a difference cannot play a role in the PAL task. In this assay a possible–though admittedly feeble–explanation could be if STZ would cause a higher anxiety state in Long-Evans than in white rats, which would resulted in a higher sensitivity to punishment allowing stronger fear memory formation.

In the NOR test, control but not STZ-treated animals explored significantly more the unfamiliar object, and the discrimination index of the STZ group was significantly lower. These findings are in accordance with those in the literature (Lu et al., 2017; Hashemi-Firouzi et al., 2018; Moreira-Silva et al., 2018; Wu et al., 2018; Zappa Villar et al., 2018). In the 5-CSRTT paradigm STZ-treated animals showed slower learning than the controls, although they were also able to acquire the task. In the Western blot measurements we found marginally significant increase in the phospho-tau/tau ratio and significant increase in the β-amyloid level in the hippocampus of animals in the STZ group compared to controls. These results are again in line with those of the literature (Correia et al., 2013; Kosaraju et al., 2013; Samy et al., 2016; Lu et al., 2017; Wu et al., 2018; Zappa Villar et al., 2018) and point out that the 3 × 1.5 mg/kg dose was sufficient to induce biochemical changes.

Overall, in EXP2, the effects of STZ were more pronounced in the NOR, 5-CSRTT, β-amyloid, and phospho-tau assays compared to EXP1, which was confirmed by the multivariate analysis. However, we still get no difference in the two key tests, MWM and PAL. We can conclude that some tests may be more sensitive to treatment (prominently the NOR task), while the aversively motivated learning tasks (PAL and MWM) still remained insensitive to the effect of STZ despite the elevated dose. Thus, our findings suggest that Long-Evans rats are likely less sensitive to STZ treatment. As this strain is crucial in our test system, we continue experimenting in it with the STZ treatment. We plan to apply the 3 × 1.5 mg/kg dosing of STZ in trained, experienced animals and also in aged rats. A possible modification of the model could be the injection of 3 × 2 mg/kg dose or administration of STZ via osmotic minipump, to ensure a continuous and longer lasting exposure to the drug.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Government Office of Pest County, Hungary; resolution number:PE/EA/785–5/2019.

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AUTHOR CONTRIBUTIONS

AG: designing the study, designing and carrying out experiments (behaviour), performing surgery, evaluating the results, statistical analysis, writing the article. BH: designing and carrying out experiments (Western blot), evaluating the results, statistical analysis, writing the article. AE: designing and carrying out experiments (behaviour), evaluating the results, reviewing the article. BT: carrying out experiments (behaviour). BV: carrying out experiments (behaviour). ZZ: designing and supervising experiments (Western blot), evaluating the results. IG: funding acquisition, designing the study, designing and supervising experiments (behaviour), statistical analysis, writing the article, reviewing and editing the article.

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Corrigendum: Intracerebroventricularly Injected Streptozotocin Exerts Subtle Effects on the Cognitive Performance of Long-Evans Rats

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KEYWORDS

Alzheimer disease model, STZ icv., cognitive test battery, learning impairment, β-amyloid, phospho-tau

A Corrigendum on
Intracerebroventricularly injected streptozotocin exerts subtle effects on the cognitive performance of long-evans rats

by Gáspár A, Hutka B, Ernyey AJ, Tajti BT, Varga BT, Zádori ZS and Gyertyán I (2021) Front. Pharmacol. 12:662173. doi: 10.3389/fphar.2021.662173

In the published article, there was an error in Figure 8 as published. The results of a mistaken measurement were shown in Figures 8A, B. Consequently, the numerical values of the *t*-test comparing the phospho-tau/total tau ratios in the control and STZ treated groups in EXP1 and EXP2 are inadequate. The corrected Figure 8 and its corrected caption appear below:

Furthermore, the name and catalogue number of the phospho-tau antibody in the Western Blot section was erroneous. As such, a correction has been made to "Methods and materials, Western Blot." The sentence previously stated:

"Membranes were incubated with primary antibodies against PHF1 (sc515013, 1: 1,000, Santa Cruz Biotechnology, Santa Cruz, CA, United States)..."

The corrected sentence appears below:

"Membranes were incubated with primary antibodies against PHF-13 (sc32275, 1: 1,000, Santa Cruz Biotechnology, Santa Cruz, CA, United States)..."

The numerical values of the multivariate analysis of variance results were also inadequate. Therefore, a correction has been made to "Results, Multivariate analysis of variance." The sentence previously stated:

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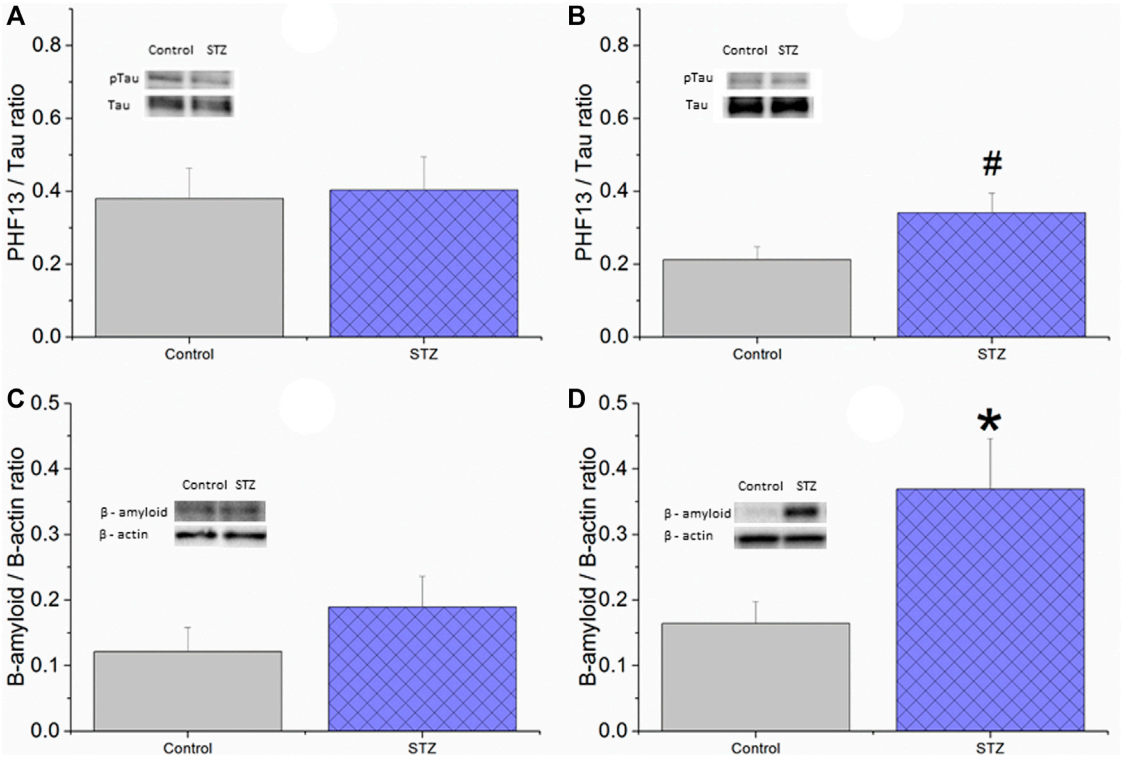

FIGURE 8
The effect of icv. STZ or citrate buffer (control) treatment on the tissue protein levels of phospho-Tau **(A,B)** and β-amyloid **(C,D)** in EXP1 **(A,C)** and EXP2 **(B,D)** measured by Western blot. Means ± SEM values are shown. There was no significant difference between the groups in phospho-tau/tau ratio in EXP1 (unpaired *t*-test: t (14) = −0.19, ns) **(A)** whereas a trend for an increase in the STZ group can be seen in EXP2 (unpaired *t*-test: t (22) = −2.012, $p = 0.06$) **(B)** Also, there was no significant difference in β-amyloid level in EXP1 (unpaired *t*-test: t (13) = −1.13, ns) **(C)** while significantly elevated β-amyloid level was found in the STZ-treated group in EXP2 (unpaired *t*-test: t (20) = −2.45, $p < 0.05$ **(D)** *<0.05, #<0.10.

"The difference between the control and STZ groups was significant in EXP2 (Wilks λ = 0.391, F(4,13) = 5.054; $p = 0.011$) whereas it was not significant in EXP1 (Wilks λ = 0.750, F(4,7) = 0.583; $p = 0.685298344$)."

The corrected sentence appears below:

"The difference between the control and STZ groups was significant in EXP2 (Wilks λ = 0.397, F(4,13) = 4,931; $p = 0.012$) whereas it was not significant in EXP1 (Wilks λ = 0.583, F(4,6) = 1.072; $p = 0.446$)."

The authors apologize for these errors and state that this does not change the scientific conclusions of the article in any way. The original article has been updated. This is a provisional file, not the final typeset article

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Performance of the intracerebroventricularly injected streptozotocin Alzheimer's disease model in a translationally relevant, aged and experienced rat population

Attila Gáspár, Barbara Hutka, Aliz Judit Ernyey, Brigitta Tekla Tajti, Bence Tamás Varga, Zoltán Sándor Zádori & István Gyertyán✉

The intracerebroventricularly (icv) injected streptozotocin (STZ) induced brain state is a widely used model of sporadic Alzheimer-disease (AD). However, data have been generated in young, naive albino rats. We postulate that the translationally most relevant animal population of an AD model should be that of aged rats with substantial learning history. The objective of the study was thus to probe the model in old rats with knowledge in various cognitive domains. Long-Evans rats of 23 and 10 months age with acquired knowledge in five-choice serial reaction time task (5-CSRTT), a cooperation task, Morris water-maze (MWM) and "pot-jumping" exercise were treated with 3 × 1.5 mg/kg icv. STZ and their performance were followed for 3 months in the above and additional behavioral assays. Both STZ-treated age groups showed significant impairment in the MWM (spatial learning) and novel object recognition test (recognition memory) but not in passive avoidance and fear conditioning paradigms (fear memory). In young STZ treated rats, significant differences were also found in the 5CSRTT (attention) and pot jumping test (procedural learning) while in old rats a significant increase in hippocampal phospho-tau/tau protein ratio was observed. No significant difference was found in the cooperation (social cognition) and pairwise discrimination (visual memory) assays and hippocampal β-amyloid levels. STZ treated old animals showed impulsivity-like behavior in several tests. Our results partly coincide with partly deviate from those published on young, albino, unexperienced rats. Beside the age, strain and experience level of the animals differences can also be attributed to the increased dose of STZ, and the applied food restriction regime. The observed cognitive and non-cognitive activity pattern of icv. STZ in aged experienced rats call for more extensive studies with the STZ model to further strengthen and specify its translational validity.

Development of animal models with better translational relevance is essential for better understanding of Alzheimer's disease (AD) and for more efficient drug development as well. Regrettably, no new cognitive enhancers have been found in the last 20 years mostly due to lack of efficacy[1,2]. Disease modifying drugs most advanced in the pipeline—but finally failed—targeted the β-amyloid cascade[3] and relied on transgenic mouse models of the familial form of the disease[4]. The Intracerebroventricularly (icv) injected streptozotocin (STZ) represents an alternative approach as it is a widely used model of sporadic AD. The construct validity of the icv. STZ model is based on the induced insulin resistant brain state[5] which gives rise to 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 at 6 months[6,7].

Our group established a rodent test system in which the animals acquire several types of cognitive tasks and then maintain their performance in regular training sessions[8–10]. Hereby we create a population with "widespread

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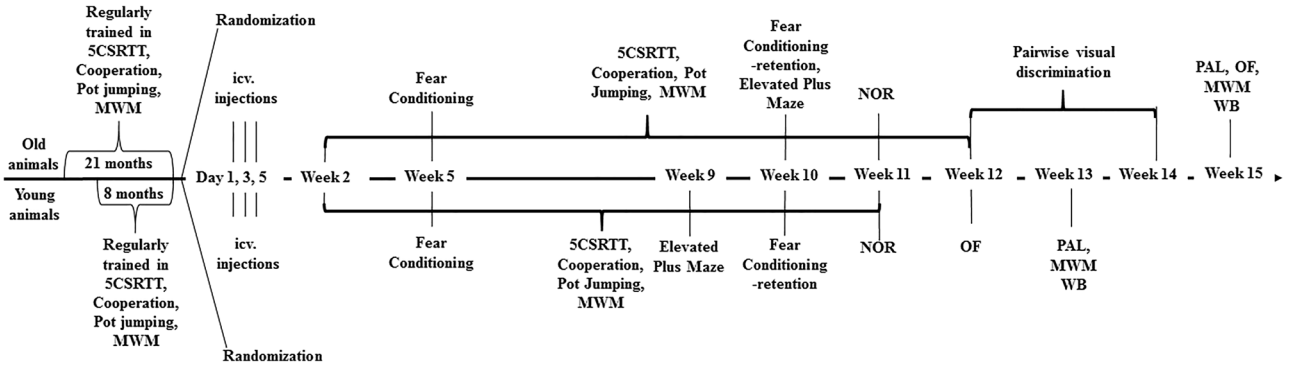

Figure 1. Timeline of the experiments (*icv* intracerebroventricular, *STZ* streptozotocin, *NOR* novel object recognition, *5CSRTT* five choice serial reaction time task, *OF* open field, *PAL* passive avoidance learning; *MWM* Morris water-maze, *PD* pairwise visual discrimination, *WB* western blot).

knowledge" which better models the human population than naïve or freshly taught animals. This "widespread knowledge" is then impaired with various kinds of interventions to create a 'patient population', amenable to test cognitive enhancers on. We use Long-Evans rats as experimental subjects because of their well-known good learning ability, which is an essential requirement in the system[11–15]. Integration of the STZ induced insulin resistant brain state model to our specific test system could result in a model which well imitates the human cognitive decline.

As the icv. STZ model has been almost exclusively used in young albino rat strains, in our previous experiments, we already examined the effect of STZ in young naïve Long-Evans rats and found that an increased dose was required to elicit subtle AD-like symptoms[16]. These results suggest that there may be specific differences between strains.

In this study, we examined the effect of icv. STZ in Long-Evans rats with widespread knowledge in two different age groups (old and young), since theoretically, old experienced animals are translationally the most relevant population for the experimental investigation of AD. For logistical reasons the two age groups were studied in two separate experiments.

Methods

Animals. Twenty-nine 23 months old and twenty-four 10 months old male Long-Evans rats ('old' and 'young' animals, respectively; obtained from Janvier Labs, Le Genest-Saint-Isle, France) were used in this study. Animals were kept three in a cage with paper tubes and wooden bricks as environmental enrichment tools under reverse light dark cycle (dark phase from 4 am to 4 pm). Animals had a restricted food access: 45 g of food was supplied for three rats before the end of the dark phase. We kept the animals under this regime because food restriction has repeatedly been shown to be healthier than *ad lib* feeding, slow the aging process and the age-associated increase in mortality rate[17–20] as well as prolong cognitive functioning[21–23]. Furthermore, this regime made the animals motivated to work in the food-rewarded tasks on the following day. Food restriction was suspended for the period of icv. STZ injections and one week recovery thereafter when rats had free access to food. Drinking water was available ad libitum over the whole course of the experiment. The animals were intensively handled before and during the experiments and were regularly trained in several learning paradigms for 21 months (old animals) or 8 months (young animals), these are specifically described below and in the Supplementary material. At the end of the post treatment behavioral measurements, they were anaesthetized by isoflurane and decapitated to remove their hippocampus for the western blot measurements. The experiments were authorized by the regional animal health authority in Hungary (resolution number PE/EA/85–5/2019) and conformed to the Hungarian welfare law and the EU 63/2010 Directive and ARRIVE guidelines.

Experimental design. The flow of the experiments is shown in Fig. 1. Sample size determination for young rats was carried out by power analysis centered on the novel object recognition test since it has got the largest standard deviation among the behavioral assays. We obtained values from the G*Power 3.1.9.7 software[24], (n = 12) as the group size for young animals. From the available 29 old animals we assigned 15 to the control and 14 to the STZ group taking into account possible losses because of their age. Based on the baseline results in the cognitive assays the animals were randomly assigned to the treatment groups (STZ or vehicle) (Fig. 1). In the experiment with the old animals, two STZ-injected and three control rats did not recover from anesthesia. We lost four additional animals from the STZ group in the course of the experiment. Two died at weeks 2 and 11, while two others were euthanized due to poor health at weeks 9 and 11.

Intracerebroventricular streptozotocin treatment. Icv. injection of STZ was carried out according to Gáspár et al.[16]. 4.5 mg/kg STZ (Sigma-Aldrich, St. Louis, MO, United States) 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 (Santa Cruz Biotechnology, Santa Cruz, CA, United

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States). The control groups received vehicle treatment in both experiments. Rats anaesthetized via a mixture of ketamine (80 mg/kg) (Produlab Pharma B.V. Raamsdonksveer, Netherlands) and xylazine (10 mg/kg ip.) (Produlab Pharma B.V. Raamsdonksveer, Netherlands) during the first drug administration and isoflurane (4% in pure oxygen) (CP-Pharma GmbH, Burgdorf, Germany) during the 2nd and 3rd surgeries. The icv. coordinates were: 0.72 mm posterior to bregma, 1.5 mm lateral to sagittal suture, 3.6 mm ventral of the surface of the brain[25].

Behavioral assays. *Morris water-maze (MWM).* The apparatus[26] was a black circular pool (diameter 190 cm, depth 60 cm) filled with water (38 cm, 23 ± 1 °C) and containing a non-visible round escape platform (10 cm diameter) placed 0.5 cm below the water surface. The platform was located in one of the four quadrants (south-east (SE), south-west (SW), north-east (NE), north-west (NW)), 40 cm from the edge of the pool. On the wall of the experimental room extra-maze cues were placed to facilitate the orientation during swimming. The learning session consisted of 3 daily trials. At the start of a trial the rat was placed into the pool at one of the four possible start points and had 3 min to find the hidden escape platform. When the animal didn't find it, it was gently guided to the platform and allowed to climb onto it. Rats could spend 30 s on the platform then were taken out, dried and replaced in their home-cage. The interval between the trials was 30 min. Escape latency was measured and swimming path was recorded by Smart v3.0 video tracking system software (Panlab, Barcelona, Spain). Rats learned the task with the platform fixed at the SE quadrant, then they received monthly maintenance training sessions in which the location of the platform was rotated around the four quadrants from session to session.

5-choice serial reaction time task (5CSRTT). The 5CSRTT device[27] consisted of a 31 × 35 × 34 cm test box (cat. no. 259920) (TSE Systems, Bad Homburg vor der Höhe, Germany). The boxes were equipped with 5 nose-poke modules on the back wall and with a magazine at the front wall. During the task, after 5 s inter-trial interval, in one randomly selected nose-poke module a 1 s long stimulus was presented and the animal had to nose-poke into the signalled hole. The animal made a correct response if nose-poked into this hole during the stimulus presentation or within 5 s afterwards (limited hold). Correct responses were rewarded with a pellet delivered into the magazine. Nose-poke into the magazine initiated the next trial. The animal made an incorrect response if nose-poked into one of the holes where the stimulus was not presented. An omission response was recorded when the rat did not make any nose-poke up to the end of the limited hold. Incorrect responses and omissions were followed by 5 s time-out punishment, when the house light was turned off. After the time-out, the house light was set back and the rat could start the next trial by nose-poking into the magazine. The animal made a premature response, if nose-poked into any of the holes during the inter-trial interval. These responses were also punished with time-out. Length of a daily test session was 20 min. The outcome parameters were the percentages of correct, omission and premature responses and accuracy defined as $\left(\frac{total\ correct\ responses}{total\ correct\ responses + total\ incorrect\ responses} \times 100\right)$.

Pot jumping. The experiment was conducted according to Ernyey et al.[28]. In the MWM tank 12 flower pots (16 cm high and 10 cm wide at the bottom) were placed upside down forming a circle. Distance between the centers of the adjacent pots gradually increased from 18 to 46 cm in anticlockwise direction. The tank was filled with 6 cm deep water 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 behavior was observed and recorded with a video camera system. Outcome parameters were the longest interpot distance jumped over and the number of passes.

Cooperation task. Social memory was measured in a cooperation task modified after Kozma et al.[29]. in a 30 × 24 × 21 cm Skinner box (MedAssociates, VT, USA). 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. One of the animals had to nose poke in to the nose poke module for 3 s, when it activated the lever press module at the opposite side. The other animal had to push the lever, as a result of which they received a reward pellet and started a new trial. The task was unsuccessful if one of the steps was missing. An omission response was recorded when the rats did not make any nose-poke or lever press. Out of sequence and incorrectly timed responses were punished with 5 s timeout. Length of a daily test session was 20 min.

Fear conditioning. The test device was a sound-proof shocking chamber (26 × 26 × 30 cm) (Ugo Basile, Gemonio, Italy) in which the fear-behavior of the animals was recorded with an infrared video camera controlled by the software EthoVision v13.0 (Noldus, Wageningen, Netherlands). The experiment, based on Varga et al.[30], consisted of one acquisition and two retention trials (24 h and 1 month later). Duration of each session was 5 min. During the acquisition trial, the rats received 5 mild foot-shocks as unconditional stimulus (0.6 mA, 1 s), the delay between shocks was 60 s. The shocks were preceded by a combination of continuous sound (65 dB, 3 kHz) and flickering light (1 Hz) conditional 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.

Novel object recognition (NOR). The test apparatus[31] was a 48 × 48 × 42 cm box with bedding material on the bottom where the behavior of the animals were recorded by a video camera system. The assay consisted of an acquisition trial and a retention trial. In the acquisition trial, the rats had 3 min to explore two identical objects in the box. After a delay of 80 min, in the retention trial one of the objects was changed to a novel one and the ani-

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mals had 3 min again to explore them. The recognizable objects were a plastic bottle filled with gravel and a glass bottle filled with blue dye solution. Exploration time of each object was the registered parameter. Recognition memory was characterized by the discrimination index, $DI = \frac{new\ object - old\ object}{new\ object + old\ object}$. Rats that explored the objects for less than 10 s or explored only one of the two objects in any of the trials were excluded from the evaluation (one animal from the control group and one rat from the STZ group among the young animals).

Pairwise visual discrimination. The task[32] was carried out in a touchscreen apparatus (Campden Instruments Ltd., Lafayette, IN, USA, cat. no. 80604). The boxes were equipped with a touch screen at the front and with a magazine at the back wall. The touchscreen wall can be divided into two sections using a cover panel. Subjects (old animals) were trained to discriminate between two images (one was correct, the other was incorrect) presented randomly in the left or right window of the touchscreen. Nosepoking the correct image was rewarded with a pellet. Choosing the incorrect image led to 5 s time out, when the houselight turned on. Entering and exiting the food magazine initiated the next trial i.e. appearance of the two images. Length of a daily test session was 30 min. Number of completed trials, correct and incorrect responses were registered by ABET II touch v2.15 software.

Passive avoidance learning (PAL). The type of the experiment was a step through passive avoidance test[33]. The apparatus consisted of a light and a dark chamber separated by a guillotine door. The test consisted of two parts, the acquisition trial and 24 h later the retention trial. During the trials the rats were placed into the light chamber and 30 s later the door opened and the animal could cross into the dark chamber. In the acquisition trial the animals had 180 s (cut off time) to enter the dark compartment of the device, whereas at the retention trial the cut off time was 300 s. When the rat passed through to the dark side, the door closed and after a 3 s delay a mild foot shock (0.6 mA, 3 s) was delivered. The animal was left in the dark compartment for an additional 5 s after the shock. The measured parameters were entry latencies into the dark compartment in the acquisition and the retention trials. Animals which did not cross to the dark chamber at the acquisition trial were excluded from the experiment (two rats from the STZ group in the young group).

Elevated plus maze (EPM). The apparatus[34] consisted of four arms (50 × 15 cm), two opened and two closed arms, the latter with 40 cm high walls. The arms were connected in a central square (15 × 15 cm). The entire maze was elevated 52 cm from the floor. The animals were placed in the central square, facing one of the open arms and had 300 s to explore the maze. The behavior of the rats were recorded by a video camera system. The measured parameters were the times spent in the open arms and the entry numbers to the arms. One rat from the young STZ group which did not moved from the central square was excluded from the experiment.

Open field (OF). The test apparatus[35] was a 48 × 48 × 40 cm box with 30 × 30 infrared beam net where the horizontal and vertical behavior of the animals were recorded by automated Conducta moti-meter system (Experimetria, Budapest, Hungary). The animals placed in the center of the apparatus and their behavior was recorded for 30 min. Analyzed parameters were the ambulation time, local movement time and immobility time.

Western blot (WB). After the behavioral tests, the animals were decapitated, their brains were removed and both hippocampi were dissected then frozen and stored at − 80 °C. Membranes were incubated with primary antibodies (obtained from Santa Cruz Biotechnology, Santa Cruz, CA, United States) against Phospho-Tau (p-tau) (PHF-13, sc32275)[36], Tau (sc32274)[37] and β-Amyloid (sc28365)[38] 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.

Data and statistical analysis. Group means ± standard error were calculated and significance was determined by unpaired t-test (NOR, PAL, EPM, OF, WB), single sample t-test (NOR), repeated measures ANOVA (MWM, pot jump, 5CSRTT, cooperation, fear condition, pairwise visual discrimination) or one-way ANOVA (WB) using the Statistica 13.5.0.17 software package (TIBCO Software Inc.). In tasks involving repeated measurements, data of animals lost (died or euthanized) during the course of the experiment were handled according to the last observation carried forward method. The number of old animals actually taking part in a measurement is shown in the corresponding figure legend.

Results

Morris water-maze (MWM). STZ-treated rats needed significantly longer time to find the hidden platform in both experiments. The difference was maintained throughout the whole measurement period except at day 4 in young rats, when the treated animals performed similarly to controls though still significantly worse than at their own baseline (Fig. 2A,B).

5-choice serial reaction time task (5CSRTT). Old STZ-treated rats produced significantly more premature responses than that of the controls in the post-injection period from Week 2 to Week 12 (Fig. 3C). There was no significant difference between the groups in the percentage of correct responses and omissions (Fig. 3A,B). Response accuracy was significantly lower in the 'STZ' group on the first post treatment occasion, however, this difference was not detectable on additional measurement days (Fig. 3D). In young rats, STZ-treated animals showed significantly reduced correct responses and increased omissions up to Week 6 (Fig. 3E,F) with preserved

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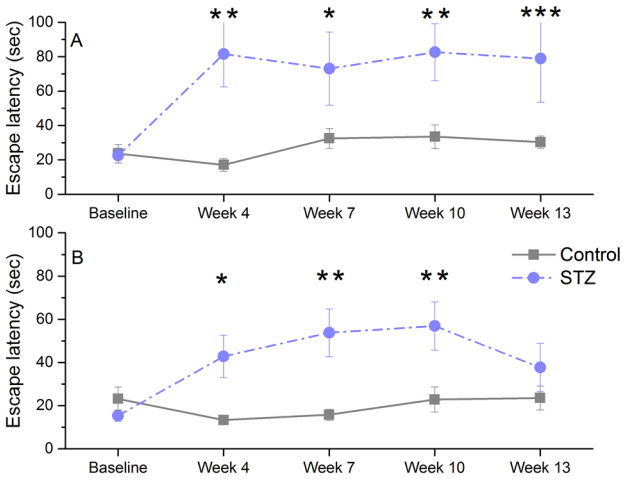

Figure 2. Learning performance of icv. STZ-injected ('STZ') and vehicle-treated ('control') rats in the Morris water-maze at various time points post-injection. Means ± SEM of daily latency values are shown. (**A**) Results of old rats. *, **, ***: $p < 0.05$, $p < 0.01$ $p < 0.001$: significant difference between groups on days 1 2, 3 and 4 (post-hoc Duncan test following repeated measures ANOVA with significant Day × treatment interaction: $F(4, 84) = 6.13$, $p = 0.000$). (**B**) Results of young rats. *, **: $p < 0.05$, $p < 0.01$: significant difference between groups on days 1 2 and 3 (post-hoc Duncan test following repeated measures ANOVA with significant Day × treatment interaction: $F(4, 88) = 3.88$, $p = 0.006$). Group size of old STZ-treated rats: n = 11 at Week 4 and 6–8, n = 10 at Week 9–11, n = 8 at Week 14–15.

accuracy (Fig. 3H) and unchanged premature nosepokes, except in the very last session (Week 11), when the latter was elevated compared to controls (Fig. 3G).

Pot jumping. In old rats, we could not detect significant difference between the groups in this procedural learning task either in the longest interpot distance jumped over or in the number of passes (Fig. 4A,B). In contrast, young STZ-injected rats jumped over significantly shorter distance than control rats, and made significantly less passes at the first post-treatment occasion (Week 2) (Fig. 4C,D).

Cooperation. In old rats, because of the high mortality rate the pairs were broken and it was not possible to evaluate the data. In young rats, there was no significant difference between the learning performances of the two groups (Fig. S1).

Fear conditioning (FC). There was no significant difference between the behavior of the animals in acquisition trials in any of the experiments. In old rats, STZ-treated animals had longer freezing time compared to the controls in the retention trials (24 h and 1 month later) but the difference was not statistically significant (repeated measures ANOVA, treatment effect: $F(1, 20) = 4.08$, $p = 0.057$; treatment*trial effect: $F(2,40) = 3.06$, $p = 0.058$). In young rats, there was no significant difference in retention trials either (Table 1A).

Novel object recognition (NOR). We found significant difference between the groups in the DI parameters in both experiments. In old rats, control animals showed a DI (0.19) significantly different from zero (no discrimination) whereas the DI of STZ-treated rats (0.06) did not differ from zero (Fig. 5A). In young rats, STZ-treated animals had a significantly lower DI (0.05) compared to the controls (0.25) (Fig. 5B).

Pairwise visual discrimination in old rats. The STZ-treated animals made a significantly higher number of incorrect responses (Fig. 6B) and their number of completed trials were also significantly higher compared to the controls (Fig. 6D). Nevertheless, there was no difference between the two groups in the percentage and number of correct responses (Fig. 6A,C).

Passive avoidance learning (PAL). In old animals, there was no significant difference between the learning performances of groups either in acquisition or retention trials (Table 1B). In young animals, during the acquisition trial, the STZ-treated animals showed significantly longer latency to enter the dark chamber compared to the controls. In turn, there was no significant difference between the groups in the retention trial (Table 1B).

Elevated plus maze (EPM). In old rats, STZ-treated animals spent more time in the open arms and the ratio of open/total entries was significantly larger compared to the controls (Table 1C). In young rats, there was no significant difference between the two groups in either of the parameters (Table 1C).

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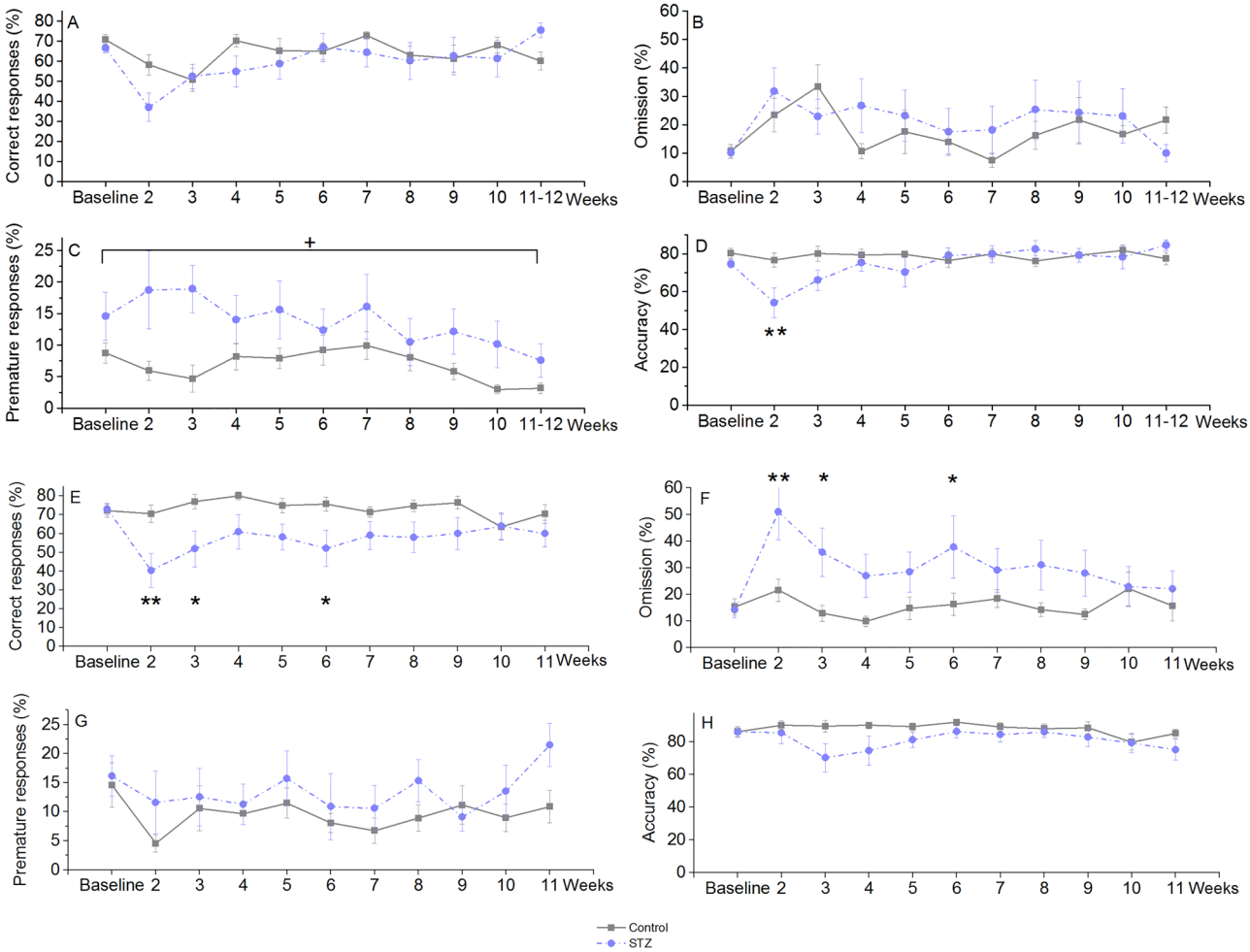

Figure 3. Learning performance of icv. STZ-injected ('STZ') and vehicle-treated ('control') rats in the 5CSRTT at various time points post-injection. Means ± SEM values are shown. (**A**, **B**, **C**, **D**) Results of old animals. +: $p = 0.023$ significant treatment effect in percentage of premature responses ($F(1, 21) = 5.98$). **: $p < 0.01$ significant difference vs control on the same day (post-hoc Duncan test following repeated measures ANOVA with significant Day × treatment interaction: $F(10, 200) = 2.53$, $p = 0.007$) for percentage of accuracy. (**E**, **F**, **G**, **H**) Results of young animals. *, **: $p < 0.05$, $p < 0.01$ significant difference between groups on the same day (post-hoc Duncan test following repeated measures ANOVA with significant Day × treatment interaction: $F(10, 220) = 2.20$, $p = 0.019$ for percentage of correct responses and $F(10,220) = 2.06$, $p = 0.029$ for omissions). Group size of old STZ-treated rats: n = 12 at Week 2, n = 11 at Week 3–9, n = 10 at Week 10, n = 8 at Week 11–12.

Open field (OF). STZ-treated rats demonstrated significantly increased activity in both experiments. Consequently, they spent significantly less time in immobility (Table 2).

Phospho-tau and beta-amyloid levels. Significant elevated p-tau/tau ratio was found in old but not in young STZ-treated rats compared to their respective controls (Fig. 7A). There was no difference in β-amyloid levels between STZ-treated and control groups in either experiments (Fig. 7B). In a separate measurement we re-assayed the β-amyloid level in the young and old experienced control rats in parallel with the samples of naïve control young animals of 5 months age studied in our previous experiment[16].We found an age-dependent increase in β -amyloid level with significant differences between the three age groups (Fig. 7C).

Discussion

In young animals, STZ-treatment impaired recognition (NOR) and spatial memory (MWM) and attention (5-CSRTT). However, the latter effect was transient, as it passed by the end of the experiment suggesting that the previously acquired knowledge could compensate the detrimental effect. Impaired procedural memory (pot jump test) was also found in young STZ treated rats. In contrast, there was no significant difference between the control and STZ-treated groups in the PAL and FC tests, and in the cooperation paradigm; that is, STZ treatment did not affect fear memory and social learning.

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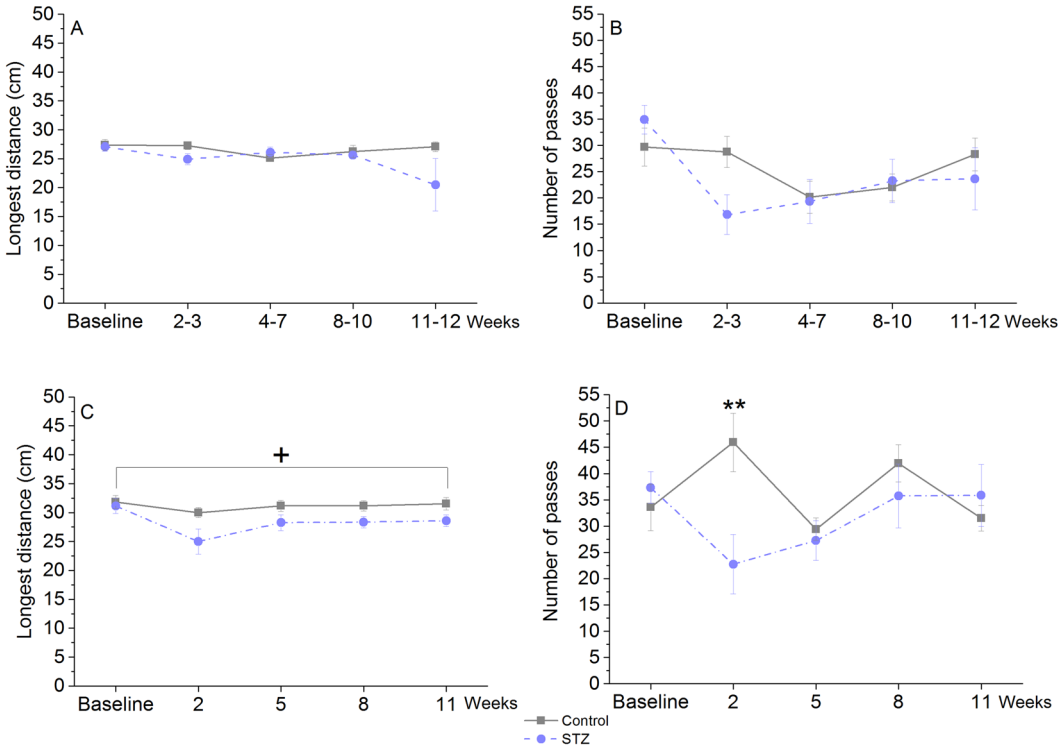

Figure 4. Performance of icv. STZ-injected ('STZ') and vehicle-treated ('control') rats in the pot jumping task at various time points post-injection. Means ± SEM of number of passes and longest distance jumped over are shown. (**A**, **B**) Results of old animals. (**C**, **D**) Results of young animals. **: $p < 0.01$ significant difference between groups on the same day (post-hoc Duncan test following repeated measures ANOVA with significant Day × treatment interaction: $F(4,88) = 5.20$, $p = 0.000$. +: $p = 0.047$ significant treatment effect in longest distance ($F(1, 22) = 4.42$). Group size of old STZ-treated rats: n = 11 at Week 2–3, 4–7 and 8–10, n = 8 at Week 11–12.

		Old rats				Young rats			
		Control		STZ		Control		STZ	
	Test	Mean	± *SEM*	Mean	± *SEM*	Mean	± *SEM*	Mean	± *SEM*
A	FC acquisition trial freezing time (s)	31.7	± *14.39*	43.3	± *11.24*	123.0	± *11.95*	84.9	± *14.22*
	FC retention trial freezing time 24 h (s)	76.9^{+++}	± *21.63*	161.2^{+++}	± *30.52*	204.5^{+++}	± *21.02*	208.4^{+++}	± *22.15*
	FC retention trial freezing time 1 months (s)	89.0^{+++}	± *25.22*	168.3^{+++}	± *32.49*	187.0^{+++}	± *25.74*	190.3^{+++}	± *22.74*
B	PAL acquisition trial entry latency (s)	33.5	± *8.23*	33.3	± *2.86*	45.7	± *9.92*	88.6*	± *13.91*
	PAL retention trial entry latency (s)	226.1^{+++}	± *35.06*	273.8^{+++}	± *7.32*	278.4^{+++}	± *17.07*	300^{+++}	± *0*
	Not entered/total number of animals	8/12		6/8		10/12		10/10	
C	EPM time spent in open arms (s)	5.3	± *3.25*	34.1	± *17.98*	19.4	± *8.40*	12.2	± *5.0*
	EPM percentage of open/total entries	3.7	± *0.022*	$21^{§}$	± *0.095*	19	± *0.063*	29	± *0.085*

Table 1. Results of icv. STZ-injected ('STZ') and vehicle-treated ('control') rats in various behavioral assays. (A) Learning performance in the Fear conditioning paradigm. 'Old rats' column: +++: $p < 0.001$ significant difference vs acquisition trial (post-hoc Duncan-test following repeated measures ANOVA with significant 'trial' effect ($F(2, 40) = 18.36$, $p = 0.000$). 'Young rats' column: +++: $p < 0.001$ significant difference vs acquisition trial (post-hoc Duncan-test following repeated measures ANOVA with significant 'trial' effect ($F(2, 44) = 43.54$, $p = 0.000$). Group size of old STZ-treated rats: n = 11 at retention trial 24 h and n = 10 at 1 month. (B) Passive Avoidance Learning. 'Old rats' column: +++: $p < 0.001$ significant difference vs acquisition trial (post-hoc Duncan-test following repeated measures ANOVA with significant 'trial' effect ($F(1, 18) = 89.44$, $p = 0.000$). 'Young rats' column: *: $p < 0.05$ significant difference vs control: unpaired t-test, $t(20) = -2.56$, effect size: 1.15; +++: $p < 0.001$ significant difference vs. acquisition trial (post-hoc Duncan-test following repeated measures ANOVA with significant 'treatment' ($F(1, 20) = 5.41$, $p = 0.030$) and 'trial' ($F(1, 20) = 397.41$, $p = 0.000$) effects. Group size of old STZ-treated rats: n = 8. (C) Elevated plus maze results. 'Old rats' column: § $p = 0.042$ significant difference vs control (Mann–Whitney U-test, U = 29; because of variance inhomogeneity non-parametric test was used), effect size: 0.91. Group size of old STZ-treated rats: n = 10. Group size of young STZ-treated rats: n = 11.

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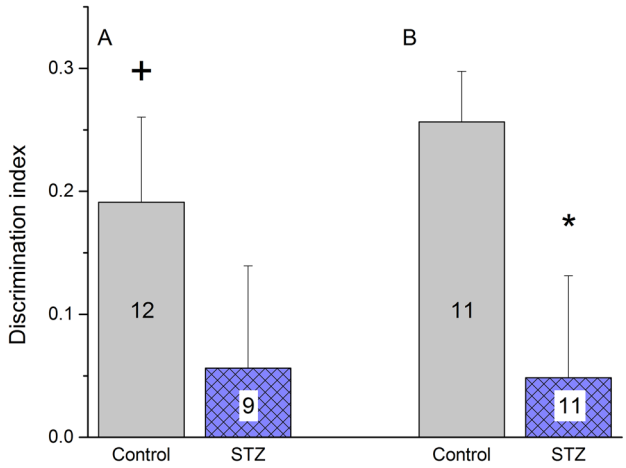

Figure 5. Novel object recognition performance of icv. STZ-injected ('STZ') and vehicle treated ('control') rats at Week 11 post-injection. Columns show means ± SEM values of discrimination index. Numbers inside the columns indicate the number of animals. (**A**) Results of old animals. +: $p < 0.05$ vs zero, singe sample t-test, control: $t(11) = 2.76$, STZ: $t(8) = 0.67$, ns.), effect size: 0.55.Three animals in the STZ group died before the test was carried out (**B**) Results of young animals. *: $p < 0.05$ vs control, unpaired t-test, $t(20) = 2.24$), effect size:0.96. Two rats were excluded from the evaluation according to the criteria described in the Methods section.

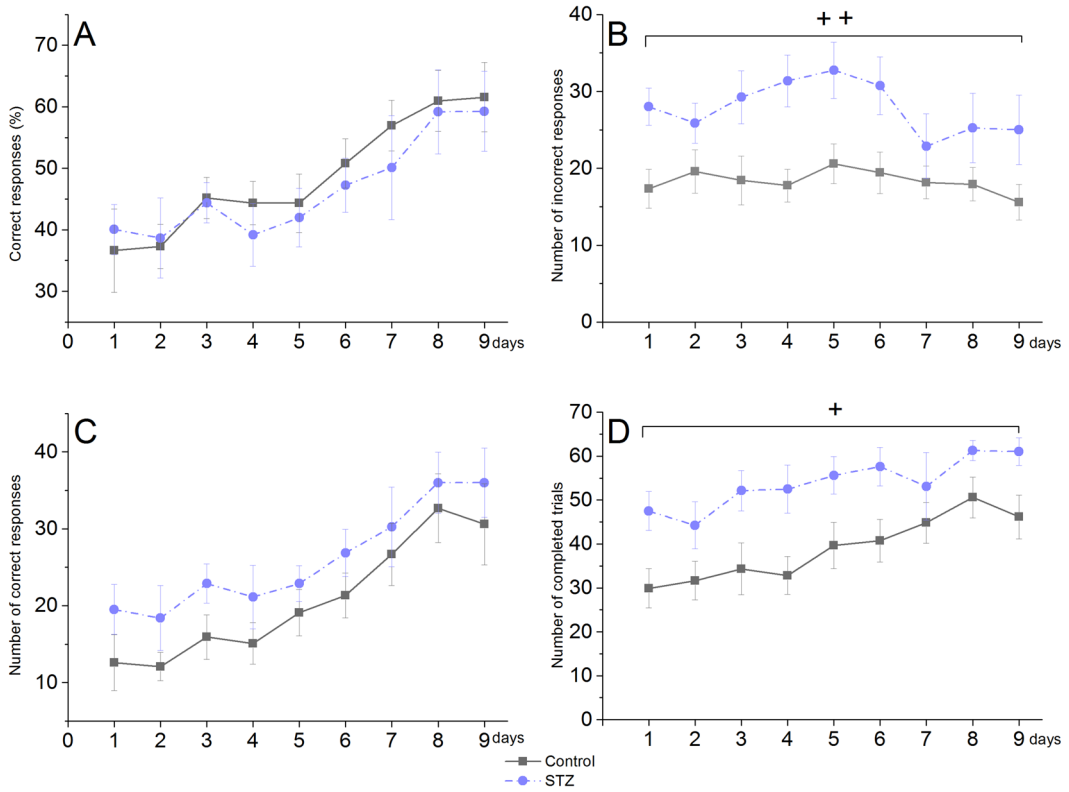

Figure 6. Pairwise visual discrimination performance of icv. STZ-injected ('STZ') and vehicle-treated ('control') old rats in a touchscreen apparatus in the post-injection period of Week 12–14. Means ± SEM values are shown. (**A**) Percentage of correct responses (**B**) Number of incorrect responses. ++: $p = 0.005$ significant treatment effect ($F(1,18) = 10.28$). (**C**) Number of correct responses. (**D**) Number of completed trials. +: $p = 0.018$ significant treatment effect ($F(1,18) = 6.83$). Group size of old STZ-treated rats: n = 8.

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	Old rats				Young rats			
	Control		STZ		Control		STZ	
Test	Mean	± *SEM*	Mean	± *SEM*	Mean	± *SEM*	Mean	± *SEM*
Ambulation time	188.4	± *23.23*	268.5*	± *23.62*	332.6	± *13.67*	409.2**	± *20.48*
Local movement time	727.9	± *34.40*	871.3*	± *38.34*	645.6	± *15.43*	758.5***	± *23.12*
Immobility time	833.4	± *55.32*	617.8*	± *49.67*	670.1	± *24.79*	541.4**	± *34.13*

Table 2. Open field results of icv. STZ-injected ('STZ') and vehicle-treated ('control') rats. Columns include means ± SEM values. 'Old rats' column: *: $p < 0.05$ significant difference between groups, unpaired t-test, ambulation time: $t(18) = -2.32$, effect size: 1.12, local movement time: $t(18) = -2.72$, effect size: 1.31, immobility time: $t(18) = 2.72$, effect size: −1.31. 'Young rats' column: **, ***: $p < 0.01$, $p < 0.001$ significant difference between groups; unpaired t-test, ambulation time: $t(22) = -3.11$, effect size: 1.33, local movement time: $t(22) = -4.05$, effect size: 1.73, immobility time: $t(22) = 3.05$, effect size: −1.30. Group size of old STZ-treated rats: n = 8.

		Old rats				Young rats			
	Test	Control		STZ		Control		STZ	
		Mean	±*SEM*	Mean	±*SEM*	Mean	±*SEM*	Mean	±*SEM*
A	WB pTau / Tau	0.22	±*0.03*	0.43§	±*0.08*	0.54	±*0.09*	0.91	±*0.21*
B	WB β-amyloid / β-actin	0.2	±*0.03*	0.2	±*0.05*	0.5	±*0.08*	0.5	±*0.10*

C

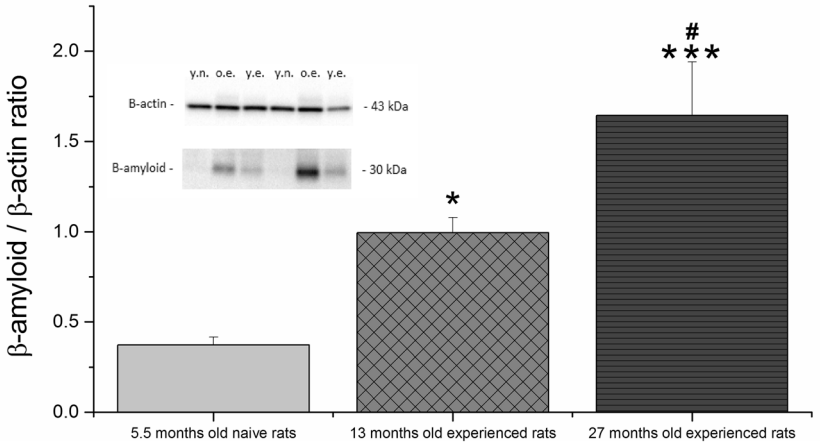

Figure 7. Results of the western blot assays. (**A**) Phospho-tau/tau ratio. 'Old rats' column: §:p = 0.016 significant difference vs control ((Mann–Whitney U-test U = 18; because of variance inhomogeneity non-parametric test was used), effect size: 1.23. Group size of old STZ-treated rats: n = 9. (**B**) β-amyloid level ns. Group size of old STZ-treated rats: n = 10. (**C**) Comparison of tissue protein levels of β-amyloid in 5 month old (young naïve), 12 month old (young experienced) and 25 month old (old experienced) rats measured by western blot. Means ± SEM values are shown. *, ***: $p < 0.05$, $p < 0.001$ significant difference vs. young naïve rats, #: $p < 0.05$ significant difference vs. young experienced rats (post-hoc Duncan test following one way ANOVA ($F(2, 24) = 10.09$, $p = 0.001$). Group sizes are 9, 11 and 11 for young naïve (y.n.), young experienced (y.e.) and old experienced rats (o.e.), respectively. The inset shows representative blots; original complete blots are presented in Supplementary material, Figs. S2-S3.

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. Biochemical markers, such as hippocampal β-amyloid and phospho-tau levels did not show significant differences either.

Looking at the results obtained in the old groups, first of all, 3 × 1.5 mg/kg STZ was more toxic to the old than to the young animals, as we lost four drug-treated rats during the post-treatment period. In old animals, similarly to young ones, STZ treatment impaired recognition (NOR) and spatial memory (MWM). However, in contrast to young rats, attention was not influenced by the treatment suggesting that the knowledge accumulated over the years became resistant to the impairing intervention. Procedural memory was also not affected, although this finding may have resulted from a floor effect, as old rats moved much shorter distances than young rats in the pot-jumping test. Social memory could not be evaluated due to mortality and thus disintegration of pairs.

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Fear memory was not affected in the PAL test, and—strictly in statistical terms—neither was it in the FC test. However, STZ treated rats showed about twice as much freezing as the controls during the retention trials. It may reflect better fear memory, however (1) it would be a surprising effect of STZ and (2) is not supported by the PAL results. A major difference between the PAL and FC paradigm is that in the former the animal has control over the situation (it may choose not to enter the dangerous place) while in the latter it has not (the rat is placed into the dangerous place) and as an anticipatory reaction to the imminent danger it shows freezing. Thus, the intensity of freezing reflects not only the strength of the memory trace but also the level of anxiety related to the previously experienced shock. In the pairwise visual discrimination task the two groups showed similar learning efficiency (% correct responses) although STZ-treated rats initiated and completed a significantly greater number of trials. Results of this assay suggests that the rats' ability to acquire new knowledge was not disrupted by icv. STZ.

A peculiar and notable finding in the STZ-treated group was the increased percentage of premature responses in the 5CSRTT. This effect is interpreted as a sign impulsivity[27]. STZ treatment increased novelty-induced exploration in the open-field. Furthermore, rats from this group showed signs of decreased anxiety in the EPM test. The above results, together with the observed differences in the FC and pairwise discrimination tests, suggest that beside its cognitive effects icv. STZ exerted emotional effects as well. We interpret these findings as the compound elevated impulsivity in old rats. With this assumption, the seemingly contradictory results of the FC and EPM tests (increased vs decreased anxiety) may be explained as similar but context-dependent overreaction to the actual situation: in positive context (EPM) more courageous behavior, in negative context (FC) more fearful behavior. Also, the increased number of initiated trials in the pairwise discrimination paradigm may be interpreted as increased "interest" in the rewarded new task (positive context).

STZ differentially affected β-amyloid and phospho-tau levels: in the former no change could be observed while in the latter a significant increase was detected in the old rats.

Our results partly coincide with (MWM, NOR, phospho-tau) partly deviate (PAL, β –amyloid) from those published on young, albino, unexperienced rats.

Decreased spatial learning and memory performance in the MWM is one of the most common and characteristic effects of STZ experiments[39–55] although in some studies the impairment was only observed in the probe trial[49,56,57]. The paper of Majkutewitz et al.[53] is of particular relevance in this comparison as they—similarly to us—examined 22 months old rats and applied a protocol where the platform location changed day by day. Interestingly, in our previous study in young naïve Long-Evans rats[16] we did not find impaired MWM learning.

Impaired recognition memory in the NOR test was also detected in several studies[16,52,58–62].

Besides MWM, PAL impairment is the most common finding in the icv. STZ literature[7,40,43,44,50,54,58,59,63–67]. However, neither in this study nor in our previous experiment[16] we could detect changes in this assay.

We found only one study[60] where the effect of icv. STZ was investigated in the FC paradigm. The authors found decreased freezing response in the tone-conditioned but not in the context-conditioned version of the test.

Our findings of increased activity in the open-field are similar to those of Chen et al.[68] and Guo et al.[56] but in contrast to those of others who did not find difference in this test[54,63,64,66,67].

Anxiety level of STZ-treated animals in the EPM was measured in two studies; Ileva et al.[69] observed—in contrast to our results—increased anxiety in young STZ-treated animals, while Moreira-Silva et al.[60] found no difference from the control.

Elevated β -amyloid level is a common finding in the literature[16,44,48,58,61,62,69–72], however it was not confirmed in the present study. As in the cited studies typically 4–6 months old rats were used, a possible explanation for this discrepancy may be that the 12 and 25 months old animals of the current study already had high protein levels resulting in a ceiling effect in the STZ treatment. This assumption is backed up by our finding of a significant age-dependent increase in β -amyloid level showing appr. threefold higher levels in the 12 months old than in the 5 months old rats. For comparison: STZ could cause a 2.2 fold increase in the amount of β-amyloid in the 5 months old rats in our previous study[16]. However, as our 12 and 25 months old rats also showed cognitive impairment, the above finding suggest that the eventual effect of STZ on β -amyloid formation may not be a causative factor in its detrimental cognitive effects.

Increased phospho-tau/tau ratio was also reported in many studies[16,43,47–49,51,56,58,60–62,72]. In the current study we only detected a significant increase in the old animals, while in young rats a non-significant 68% increase was observed. Interestingly, Osmanovic Barilar et al.[73] examined STZ-treated rats of different ages and found increased phospho-tau/tau ratio in 6 and 9 months old rats but not in 12 months old ones.

Impulsive-like behavior has not been described in the literature yet, and it may be a hint for a possible direction of further investigations. It is not among the characteristic non-cognitive symptoms of AD[74,75], rather, impulsivity and disinhibition are well known symptoms of frontotemporal dementia[75–77], which lacks amyloid pathology[78,79].

Comparison of our results with those in the literature shows that the effect of icv. STZ varies in different strains, depends on the age of animals and influenced by their level of experience and learning history. However, if the method is to be considered as a dementia model then the translationally most relevant animal population should be that of (i) old and (ii) experienced rats. Up to our best knowledge the present study is the first where the effect of icv. STZ was investigated in such a population. In these animals icv STZ produced impairments in spatial and recognition memory but not in fear learning/memory, visual discrimination and social learning; however it induced impulsive-like behavior. β-amyloid level was not increased probably because of the high basal level.

Nevertheless, it would be premature to generalize these findings to the STZ-icv model as such, since beside the age and experience level of the animals several other factors differed from those common in the literature. Strain difference is one of them: Long-Evans rats are better performers in cognitive tasks than Wistar rats[11–15], and there is also a difference in local cerebral blood flow reactivity[80]. The dose of STZ applied in our study (4.5 mg/kg)[16] was greater than those used in the literature (not greater than 3 mg/kg) and we do not know whether the Alzheimer disease-like pathophysiology induced by 3 mg/kg and 4.5 mg/kg STZ-icv has the same time course

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of onset, development and progression in Long-Evans and Wistar rats. Last, cognitive performance is usually measured in ad libitum fed rats, while we applied a food restriction regime, which may have rendered the animals more resistant to the toxic effects of STZ[18,21,22]. Thus, findings of the current study together with the above discussed differences call for more extensive studies with the STZ model involving both Wistar and Long-Evans strains to further strengthen and specify its translational validity.

Data availability

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation. To request data from this study, please contact the corresponding author.

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Author contributions

A.G.: designing the study, designing and carrying out experiments (behavior), performing surgery, evaluating the results, statistical analysis, writing the article. B.H.: designing and carrying out experiments (western blot), evaluating the results, statistical analysis, writing the article. A.J.E.: designing and carrying out experiments (behavior), evaluating the results, reviewing the article. B.T.T.: carrying out experiments (behavior). B.T.V.: carrying out experiments (behavior). Z.S.Z.: designing and supervising experiments (western blot), evaluating the results. I.G.: funding acquisition, designing the study, designing and supervising experiments (behavior), statistical analysis, writing the article, reviewing and editing the article.

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Competing interests

The authors declare no competing interests.

Additional information

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