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Antioxidant treatment after epileptogenesis onset prevents comorbidities in rats sensitized by a past stressful event

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Summary

Objective. Unresolved past stressful events can leave a long-term biological imprint in some individuals and induce a state of vulnerability to epilepsy and co-morbidities, such as depression and cognitive deficits. Using an experimental model of stress-induced vulnerability to depression, we tested whether an antioxidant treatment applied after the onset of epileptogenesis was disease modifying and could prevent the occurrence of co-morbidities.

Methods. We used social defeat (SD) to trigger a state of vulnerability to epilepsy, depression and cognitive deficits in half of the SD exposed population of rats. Serum Brain Derived Neurotrophic Factor (BDNF) was collected before and one month after SD to identify the vulnerable population. One month after SD, we used repeated injections of kainic acid to trigger status epilepticus (SE). One subset of animals was treated during 2 weeks with Tempol, a strong antioxidant. Supradural 24/7 recordings were used to assess the development of epilepsy for 6 weeks following SE. We assessed spatial and non-spatial memory as well as a depression-like profile 6 weeks after SE.

Results. One month after SD, after an initial decrease, half of the animals recovered normal (pre-SD) serum BDNF levels (SDN group). The other half kept low serum BDNF levels (SDL group). At that stage, SDN and SDL animals do not present a behavioural phenotype. The SDL group was more sensitive to epileptogenic conditions than the SDN group. Following SE, the SDL group displayed accelerated epileptogenesis, a depression-like profile and severe cognitive deficits as compared to SDN rats. Transient Tempol treatment was disease-modifying, reducing the number of seizures, and prevented the development of co-morbidities in the SDL group. In keeping with the fact that SD-induced vulnerability is due to oxidative stress, we found a very large increase of oxidative stress

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6 weeks after SE in the SDL group as compared to the SDN group. Tempol treatment normalized oxidative stress in the SDL group to SDN levels.

Significance. This study illustrates the disease modifying effect of antioxidant treatment after the onset of epileptogenesis in a population rendered vulnerable by past stressful events. The transient treatment decreased seizure burden, and had long-term effects, preventing the occurrence of a depression like-profile and cognitive deficits. We propose that vulnerability to co-morbidities can be reversed after the onset of epilepsy.

Keywords: epilepsy, depression, cognitive deficits, oxidative stress, behavioral stress

Key points

- An intense stressful event can render some individuals vulnerable to epilepsy and co-morbidities (depression and cognitive deficits)
- After a double hit (social defeat and status epilepticus), half of the rats develop a severe form of epilepsy associated with co-morbidities
- A transient antioxidant treatment prevents the development of co-morbidities and decreases seizure burden in vulnerable animals

1 INTRODUCTION

Most neurological disorders, including epilepsy, are associated with co-morbidities such as depression and cognitive deficits.^{11; 15; 16} However, not all patients with epilepsy develop co-morbidities. The diathesis-stress model provides a conceptual framework to understand why some patients may develop co-morbidities and not others.¹² Past unresolved stressful events can generate a state of vulnerability.^{20; 21} Vulnerable individuals are close to the threshold for expressing a phenotype, but subsequent hits/insults are required to cross the threshold.⁸ The brain insult triggering epileptogenesis, seizure themselves and the burden/helplessness of epilepsy can act as primary or secondary hits to trigger depression.¹² The context of the present study stems from the observation that some patients report having experienced a highly stressful event before the onset of their epilepsy. If unresolved, such intense stress may have left a biological imprint in some individuals, driving them closer to the threshold of depression, cognitive deficits etc. Epileptogenesis or seizures could then act as secondary hits to make them cross the threshold. The goal of the present study is to provide the proof-of-concept in a preclinical model that rats rendered vulnerable by an intense stressful situation can be treated after the onset of epileptogenesis to prevent the occurrence of co-morbidities.

For this purpose, we use a validated double hit model.¹⁻³ The first hit is SD, an intense stressful event. Five days after SD, all animals present a post stress state characterized by a hyperactivity of hypothalamic-pituitary-adrenal axis, low serum BDNF levels, as well as the accumulation of reactive oxygen species (ROS), dendritic retraction and spine loss in the hippocampus.^{2; 3} One month after SD, animals do not have depression-like profile, but half on them still display low serum BDNF levels, ROS accumulation and neuroanatomical alterations in the hippocampus.^{2; 3} These animals are called vulnerable because after SE (second hit), they develop a severe form of epilepsy,

cognitive deficits and a depression-like profile.¹ The other group (non-vulnerable) develops a less severe form of epilepsy without a depression-like profile.¹ In our experimental model, animals only exposed to SE (i.e. no prior SD) do not develop co-morbidities, showing that the latter occur in stress-sensitized animals.¹ Although treating animals with BDNF mimetics before the second hit abolishes vulnerability and the development of co-morbidities,¹⁻³ such treatment cannot be used after SE since BDNF is directly involved in epileptogenesis.^{10;19} In order to be close to the most common clinical condition, a treatment targeting vulnerability should be provided after epileptogenesis onset. We thus turned to the mechanism of vulnerability identified in our experimental conditions, which involves ROS accumulation.³ We report that antioxidant treatment provided after triggering epileptogenesis can prevent co-morbidities, while also decreasing epilepsy severity.

2 MATERIALS AND METHODS

All materials and methods are described in online Appendix S1.

3 RESULTS

3.1 Generating vulnerable and non-vulnerable animals

We generated three groups of animals. One group was exposed to SD and received kainic acid to trigger SE (SD-SE). The other two groups were not exposed to SD and received either kainic acid (noSD-SE) or saline (noSD-noSE) (Figure 1A). In the SD group, after an acute decrease, serum BDNF returned to control levels in 53% of animals (called SDN, for SD group with normal BDNF level) and remained decreased in 47% of animals (called SDL, for SD group with low BDNF level) 2 weeks following SD (Figure 1B). At that stage, neither SDL nor SDN animals display a depression-like.¹⁻³ Vulnerable animals

correspond to the SDL group, as they develop a depression-like profile and cognitive deficits after a second hit, including SE.¹⁻³ Since serum BDNF level was the last parameter to be evaluated, all experimenters remained blind to the groups throughout the entire procedure.

3.2 Enhanced oxidative stress in vulnerable animals is normalized to non-vulnerable animal levels following Tempol treatment

We have previously shown that oxidative stress is necessary and sufficient to induce a state of vulnerability to depression and cognitive deficits following SD.³ Thus, in the SDL group, SE will occur in a context of oxidative stress. Since there is a tight relationship between ROS accumulation and epilepsy^{7; 9; 23; 25; 29}, we reasoned that SE should amplify oxidative stress in the SDL group, and tested whether the antioxidant treatment would prevent excessive ROS accumulation. We first assessed the antioxidant/detoxifying systems in animals 6 weeks after SE (Figure 1C). Superoxide dismutase (SOD) activity that catalyzes H₂O₂ production from O₂⁻ was increased in animals only exposed to SE (noSD-SE) as compared to the noSD-noSE group (Figure 1D). The activity of glutathione peroxidase, but not that of catalase, and the quantity of peroxiredoxin2 were increased in the noSD-SE group as compared to the noSD-noSE group (Figure 1E-G), indicating an activation of detoxifying/defense systems in animals with spontaneous seizures. In animals previously exposed to SD, the SDL-SE group displayed greater SOD activity and a large reduction of peroxiredoxin2 as compared to the other groups (Figure 1E-G), which would lead to a decrease of H₂O₂ clearance. Excessive ROS levels can lead to cell damages (as oxidation of lipids), signing an oxidative stress. Six weeks after SE, the noSD-SE group showed high level of lipid peroxidation in the hippocampus as compared to the noSD-noSE group (Figure 1H), showing that the activation of antioxidant defense systems was

not sufficient to prevent oxidative stress. The SDL-SE group displayed even greater levels of lipid peroxidation, indicating that vulnerability- and epilepsy-dependent oxidative stress add up to produce a stronger phenotype (Figure 1H). Previous work shows that the downregulation of Nrf2, a transcription factor that controls antioxidant defense mechanisms, is necessary and sufficient to produce a state of vulnerability to depression.³ Accordingly, Nrf2 was upregulated in the noSD-SE and SDN-SE groups, but not in the SDL-SE group (Figure 1I). Treating animals with Tempol (4-hydroxy-2, 2, 6, 6-tetramethyl piperidinoxyl), an anti-oxidant compound³⁰, starting 2 days after SE for 2 weeks normalized SOD, peroxiredoxin2, lipid peroxidation and Nrf2 in the SDL-SE group to SDN-SE and noSD-SE levels (Figure E-I). However, Tempol did not bring back values to control (noSD-noSE) levels. This is readily explained by the fact that the treatment was stopped 26 days before tissue harvest, allowing time for epilepsy-dependent ROS production and lipid peroxidation to occur. Despite this, Tempol abolished the excess in damage specifically found in the SDL-SE group (Figure 1H), in effect normalizing the Tempol treated SDL-SE group to the treated and untreated SDN-SE groups.

3.3 Tempol treatment prevents the occurrence of a depression-like profile and cognitive deficits in vulnerable animals

3.3.1 Effect of Tempol on depression-like profile

As reported before¹⁻³, neither noSD-noSE (control) nor noSD-SE (epilepsy only) animals displayed a depression-like profile as assessed with immobility time in the forced swimming test (FST), sweet water consumption and activity of the HPA axis (Figure 2A). A depression-like profile (increased immobility time, anhedonia, HPA axis hyperactivity) was found in the SDL-SE group, but not in the SDN-SE group (Figure 2A). Although Tempol treatment was provided transiently for two weeks starting two days after SE, it prevented

the occurrence of a depression-like profile in the SDL group assessed four weeks after then end of the treatment (Figure 2A). Tempol treatment had no effect in the other groups (noSD-noSE, noSD-SE and SDN-SE).

3.3.1 Effect of Tempol on spatial and non-spatial memory

As previously reported¹, neither noSD-noSE (control) nor noSD-SE (epilepsy only) animals displayed spatial memory and non-spatial memory deficits (Figure 2B). In contrast, both SDN-SE and SDL-SE animals displayed spatial memory deficits, both groups now operating at chance level (Figure 2B). In addition, SDL-SE animals, but not SDN-SE animals, also performed at chance level in the non-spatial memory test (Figure 2B). These results revealed important degradation of cognitive function in vulnerable animals. Early and transient Tempol treatment prevented the occurrence of spatial and non-spatial memory deficits evaluated 26 days after the end of Tempol treatment in SDN-SE and SDL-SE groups (Figure 2B). We conclude that transient anti-oxidant treatment can be provided following epileptogenesis onset to prevent the occurrence of subsequent co-morbidities in animals sensitized by a past history of intense stress.

3.3 Tempol treatment prevents the occurrence of a depression-like profile and cognitive deficits in vulnerable animals

Finally, we investigated the impact of the antioxidant treatment on epilepsy itself. As reported before¹, the threshold to trigger SE, the time to SE and the time for the first spontaneous seizure were lower in the SDL-SE group as compared to the noSD-SE and SDN-SE groups (Figure 3A-C). Vulnerable animals are therefore more sensitive to epileptogenic conditions (lower threshold) and spontaneous seizures occur sooner. In addition, epilepsy developed faster in the SDL-SE group (Figure 3D). Although only

applied during 2 weeks, Tempol had a strong-disease modifying effect in the SDL-SE group, slowing down epileptogenesis, with a long-term effect, decreasing seizure burden even four weeks after the end of the treatment (Figure 3D). Tempol treatment had no effect in the noSD-SE and SDN-SE groups (Figure 3D).

4 Discussion

Our results demonstrate that antioxidants can be used to reverse a vulnerability phenotype induced by a past history of stress. The experimental model we are using is particularly interesting as it reproduces an important human trait: individual variability. We and others have reported that not all animals develop co-morbidities in experimental epilepsy although being exposed to the same challenging conditions.^{1; 26} Because laboratory animals are biologically different (even from the same litter), it is not surprising to find variability in their response to external challenges. Some biological biomarkers allow differentiating animals in premorbid conditions, predicting their response to external challenges.^{1-3; 6; 22} In our experimental conditions, the retrospective analysis of serum BDNF levels enabled us to distinguish vulnerable from non-vulnerable animals. Whether serum BDNF levels can also be used in the human population as a predictive biomarker for vulnerability to depression and cognitive deficits remains to be determined.

We have previously shown that there exists a causal relationship between low BDNF levels and vulnerability to depression.³ BDNF controls the transcription factor Nrf2³, which plays a key role in controlling antioxidant defense systems.²⁴ The sustained downregulation of BDNF in the vulnerable population prevents the activation of Nrf2, leading to the accumulation of ROS, which is responsible for the state of vulnerability in our conditions.³ The scheme we wish to propose is that high levels of ROS “weaken”

neuronal networks during the premorbid stage, thus providing a favorable ground for the development of epilepsy and associated co-morbidities when the processes specific to these disorders are triggered. In such a context, SE hits brain networks already weakened by oxidative stress in vulnerable (SDL) animals. Our results show that an additive phenomenon in SDL, which express a high level of oxidative stress coming from the vulnerability state and that provided by epilepsy itself. We propose that such high ROS levels contribute to the strong phenotype found in SDL animals. In keeping with this proposal, the transient treatment with Tempol prevented the strong phenotype found in the SDL group, without affecting the epilepsy-dependent accumulation of ROS. Tempol had no effect on oxidative stress in the SDN-SE and noSD-SE groups. This result can be explained by the fact that the treatment lasted two weeks, while tissue for oxidative stress analysis was collected four weeks later.

There are conflicting reports regarding the occurrence of depression-like profile and cognitive deficits in experimental epilepsy, as phenotypes may be strain- and model-dependent^{5; 13; 14; 27}. Here we reproduced our previous results showing no co-morbidity in the epilepsy-only (noSD-SE) group.¹ The discrepancy may stem from the fact that we maintain social interaction in our experimental conditions, while most studies use individual housing in order to perform 24/7 EEG monitoring. However, social isolation (single housing) is stressful to rodents invariably resulting in oxidative stress, the production of stress hormones and inflammatory cytokines.^{4; 18; 28} Hence, a strong phenotype found in experimental animals may result from the addition from the biological alterations due to epilepsy and social isolation. Such experimental procedure is particularly useful to mimic social isolation experience by some patients with epilepsy.¹⁷ Maintaining social contact provides different results, arguably modelling a different aspect of patient diversity. This argument may also explain the difference of our results with the reported positive effect

of antioxidant treatment or targeting the Nrf2 pathway in experimental epilepsy in socially isolated animals.^{25; 29} It will be interesting to test whether the positive effects found in the latter studies are due to the removal of the social isolation component of oxidative stress by the treatment, which would be highly clinically relevant. We do not consider our results as a failure to replicate. Rather, we argue that using different experimental conditions leading to different results is the best approach to human diversity. It is interesting to note that the epilepsy-dependent accumulation of ROS in the noSD-SE group was not sufficient to trigger a depression-like profile and cognitive deficits, raising the possibility that the severity of the phenotype may be related to the level of oxidative stress. However, despite the fact oxidative stress was similar in the noSD-SE and SDN-SE groups, animals in the latter group displayed spatial memory deficits, which could be prevented by Tempol. Future studies should use more sensitive measurements of oxidative stress to determine whether the SDN group kept a biological imprint that may explain the occurrence of spatial memory deficits after a second hit.

5 Conclusions

The functional scheme we propose (unresolved past stress) may explain why some patients express a strong epilepsy phenotype and co-morbidities. Obviously, this scheme may only apply to a subset of identifiable patients, which is important in the context of personalized medicine. Our study provides the proof-of-concept that treatment can be provided after the onset of epileptogenesis to prevent the occurrence of a strong phenotype and co-morbidities. It will be interesting to test whether the framework we describe also applies to other neurological disorders associated with co-morbidities.

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Conflict of interest

The authors of the manuscript have no conflict to declare.

Figure legends

FIGURE 1: Tempol prevents the exacerbation of oxidative stress in vulnerable animals

(A) Experimental “double-hit” SD-SE protocol. Animals exposed to SD were stratified according to serum BDNF concentration as low (SDL, vulnerable group) or normal (SDN, non-vulnerable group). Fifteen day-long Tempol treatment started 2 days after SE. (B) Longitudinal follow-up of serum BDNF levels. (C) Antioxidant enzyme systems. SOD: superoxide dismutase; GPx: glutathione peroxidase; Prx: peroxiredoxin. (D) SOD was increased in epileptic animals, with a higher activity in the SDL-SE group, which was reversed by Tempol (Tp) treatment. (E-F) GPx, but not catalase, was increased in epileptic animals. (G) Prx2 was increased in epileptic animals except in the SDL-SE group, which was normalized by Tempol. (H) All epileptic animals displayed lipid peroxidation, with an exacerbation in SDL-SE animals, which was normalized by Tempol. (I) Nuclear translocation of Nrf2 was increased in epileptic animals except in the SDL-SE group. Supplementary Tables S1A and S2 A-B includes statistical analyses for BDNF and oxidative stress, respectively. € $P < 0.05$ vs noSD, \$ $P < 0.05$ vs SDN animals, † $P < 0.05$ vs noSD-noSE group, ≠ $P < 0.05$ vs noSD-noSE + Tempol (Tp) group, * $P < 0.05$ vs noSD-SE animals, a $P < 0.05$ vs SDN-SE animals, b $P < 0.05$ vs SDL-SE + Tempol (Tp) animals.

FIGURE 2: Tempol prevents the development of a depression-like profile and cognitive deficits in vulnerable animals.

(A) Depression-like profile (helplessness behaviour, decreased in sweet water consumption reflecting anhedonia, hypothalamic–pituitary–adrenal hyperactivity) only occurred in SDL-SE animals. Tempol abolished the depression-like profile. (B) Epilepsy had no impact on spatial and non-spatial memory in noSD-SE animals. SDN-SE animals displayed spatial memory deficits, whilst SDL-SE animals displayed both spatial and non-

spatial deficits. The preference for the familiar object (negative discrimination ratio after displacement or replacement) was not a sign of neophobia as the negative discrimination ratio values were not statistically different from chance levels. In contrast, all positive values were statistically different from chance levels. Tempol treatment prevented memory deficits. Supplementary Table S1 B-C includes statistical analyses. * $P < 0.05$ vs noSD-SE animals, a $P < 0.05$ vs SDN-SE animals, b $P < 0.05$ vs SDL-SE treated with Tempol (Tp) (SDL +Tp) animals.

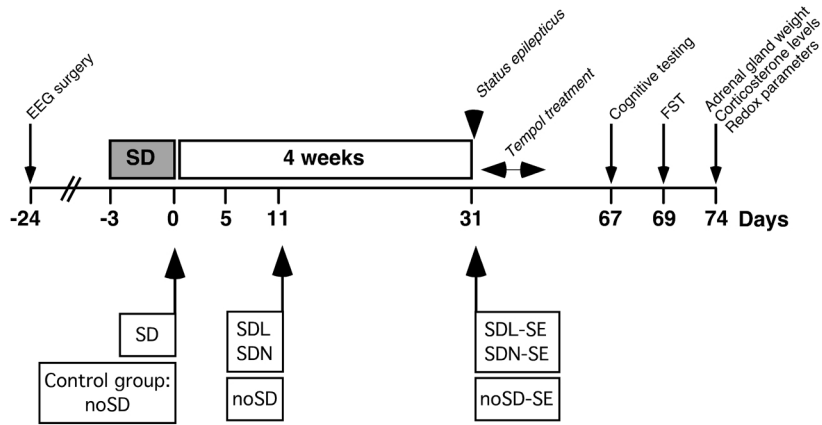
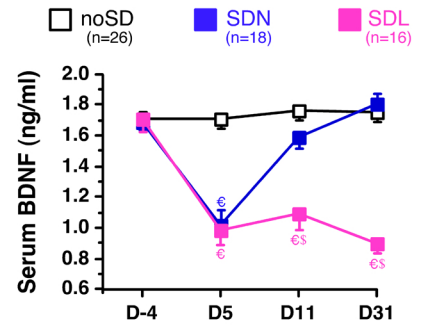
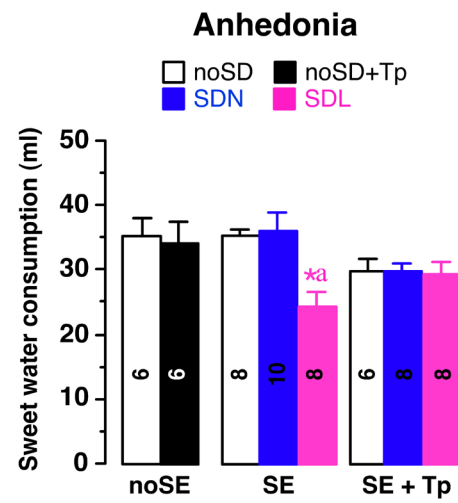
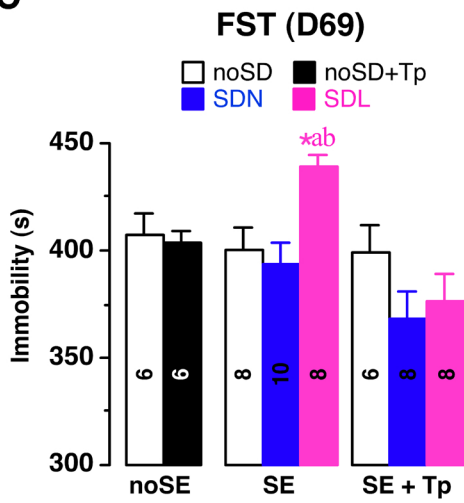
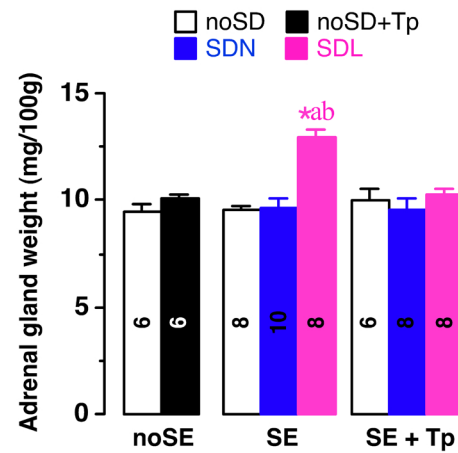
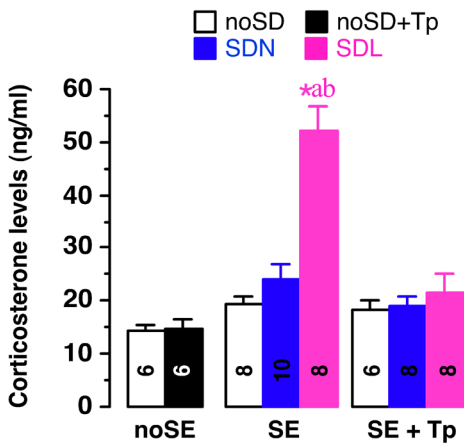
FIGURE 3: Tempol decreases seizure burden in vulnerable animals.

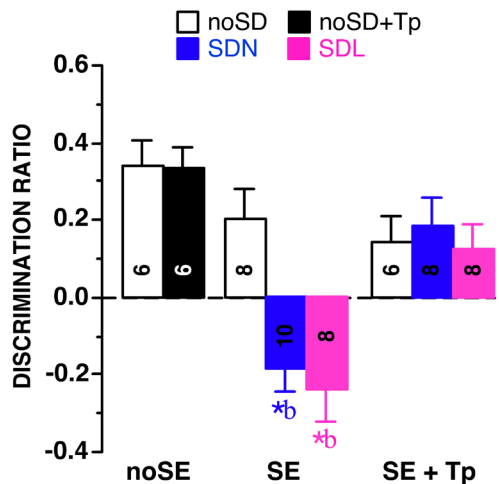
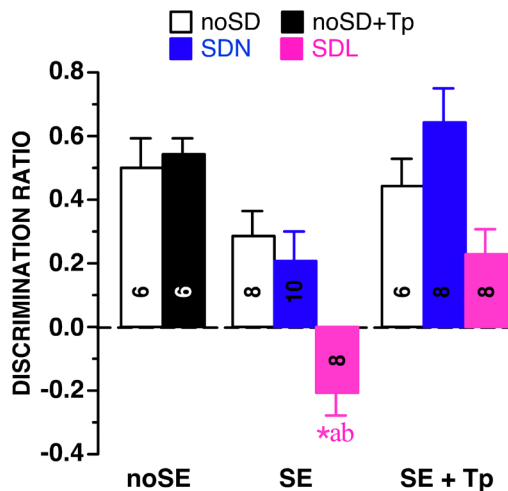
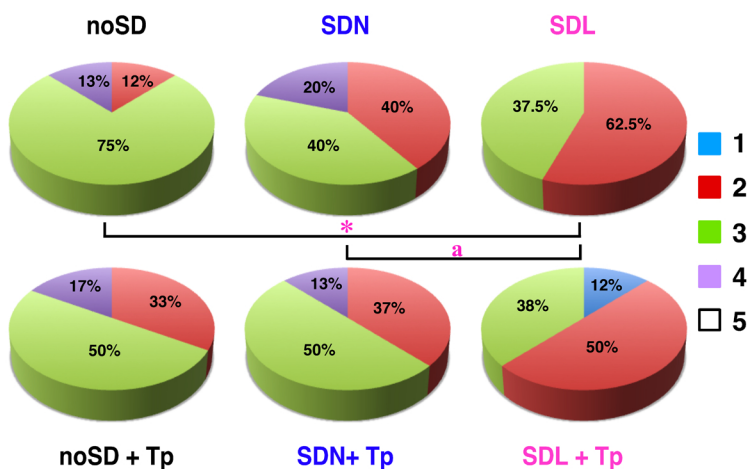
(A) Less injections of KA were needed to induce SE in SDL animals suggesting a decreased seizure threshold in this group. **(B)** The time to SE was significantly decreased in SDL animals (treated after SE and non-treated), suggesting increased seizure susceptibility in the SDL group. **(C)** The time to the first spontaneous seizure was shorter in the SDL non-treated group but recovered to SDN levels with Tempol treatment. **(D)** Epileptogenesis was markedly faster in SDL-SE animals. The cumulative number of seizures increased after the end of Tempol treatment but remained significantly lower than SDL-SE animals. Supplementary Table S1 D-E includes statistical analyses. * $P < 0.05$ vs noSD-SE animals, a $P < 0.05$ vs SDN-SE animals, b $P < 0.05$ vs SD-SE + Tempol (Tp).

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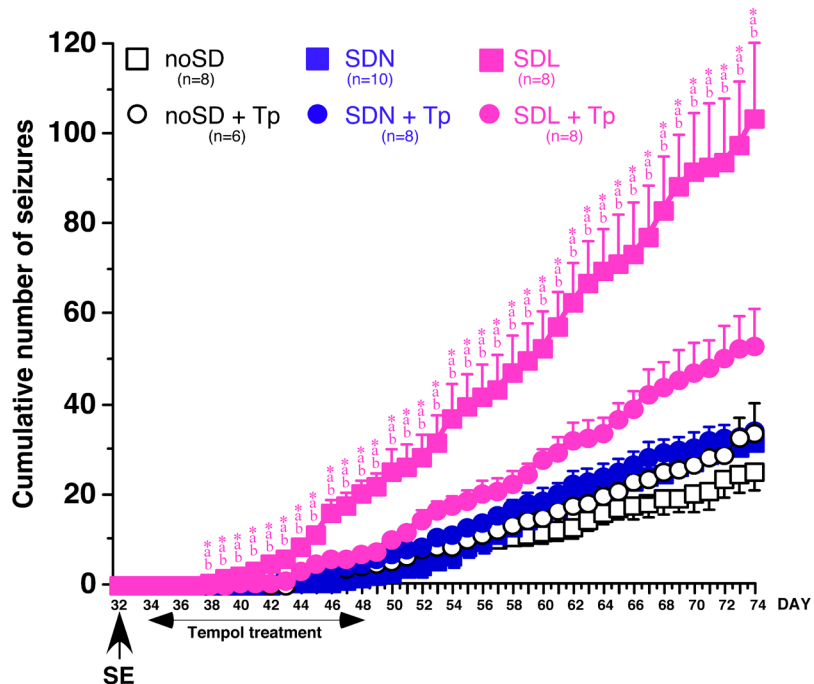
A**B****C****HPA axis (D74)****FIGURE 1**

A**SPATIAL MEMORY (D67)****NON-SPATIAL MEMORY (D67)****B****Number of injections****C****Time to SE**

	noSD	SDN	SDL
Saline	2:56 ± 0:09	3:01 ± 0:14	2:06 ± 0:08 ^{*a}
Tp	3:04 ± 0:12	2:58 ± 0:13	2:01 ± 0:09 ^{*a}

D**First spontaneous seizure**

	noSD	SDN	SDL
Saline	16.2 ± 1.5	16.9 ± 1.4	10.2 ± 1.2 ^{*a}
Tp	15.3 ± 0.8	14.0 ± 0.9	13.2 ± 1.1

E**FIGURE 2**