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## Targeting the brain under stress : selective glucocorticoid receptor modulation

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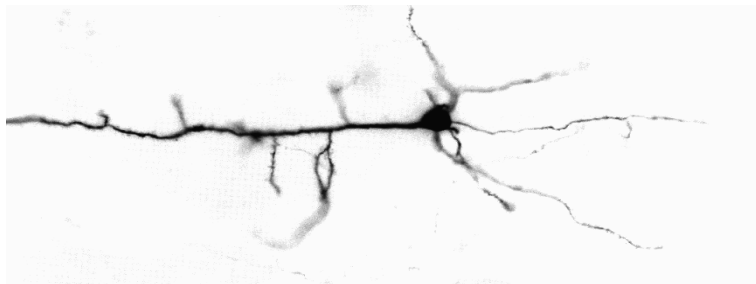
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# *Chapter*

## **General Discussion**

# **6**



## Discussion

The appropriate orchestration and expression of responses to stressors is crucial for survival and involves the coordination of multiple systems in the brain and the periphery (1-4). The HPA axis plays a central role in the regulation of stress responses via control of glucocorticoid hormone levels. Glucocorticoids, in turn, exert a wide range of effects, including effects on memory, behavior and metabolism, that are mediated by their receptors MR and GR. Importantly, glucocorticoids can block the expression of CRH in the PVN and ACTH in the pituitary, thus controlling their expression via a negative feedback loop (5, 6).

Due to their coordinating effects, the function of GR and MR must be tightly regulated in a tissue-specific fashion rather than simply follow the changes in concentration of their ligands in a uniform way. This tissue-specific regulation may take place at multiple levels, such as the expression of the receptor, the bio-availability of free ligand in plasma, the expression of enzymes that modify the ligand, the expression of other nuclear receptors, the presence of other transcription factors and the expression and availability of coregulators (4). The latter, may create a bottleneck, as competition of nuclear receptors for coregulators may be the limiting factor when multiple signals are received at the same time.

Several coregulators have been shown to be important for brain function and particularly for learning and memory and stress responses. Notable examples, apart from SRC members of the p160 family, are the coregulators of CREB CBP/p300 and pCAF, members of the CREB regulated transcription coactivator (CRTC) family, the coregulators of steroid hormone receptors RIP-140, Ube3a and proteins involved in the SWI/SNF chromatin remodeling complex (7-20). Not surprisingly, mutations or deletions of these coregulators often result in impairments in learning and memory, decreased neuronal plasticity, inappropriate regulation of stress responses or abnormal brain morphology (21).

Here, we studied the importance of coregulator recruitment in relation to stress and effects of glucocorticoids in two different ways: First, we tried to manipulate the sensitivity of the central amygdala to glucocorticoids and interfere with proper stress responses, via induction of alternative splicing of the well-described coregulator and member of the p160 family SRC-1. Secondly, with the use of novel selective ligands of the GR we tried to interfere with GR-coregulator interactions and selectively block a subset of GR-dependent functions while leaving others intact. Subsequently, we studied the effects of these ligands on stress-induced CRH expression, fear-related memory consolidation and GR-dependent gene expression in the brain *in vivo*.

### SRC-1 isoform switching in the CeA

First, we showed that AON-mediated exon skipping in the CeA is a feasible technique to modulate splicing of the *NCoA1* gene (22). We compared the immunostimulatory potential of a random 2-O'-Me ribonucleotide with a phosphorothioate backbone, which had no known targets in the murine genome or transcriptome, to saline. Our results showed no differences

between treatments in any of the markers of astrogliosis or microglia activation we used. Previous studies using similar concentrations of AONs as in our study, also reported no immunogenicity, although this might be the case for higher AON concentrations (23). In fact, the 2-O' modification used in the design of the AON may have acted as a Toll-like receptor antagonist, thus decreasing potential immunostimulatory effects (24, 25).

Secondly, we showed adequate uptake of the AONs from neurons and localization of the AONs in the cell nuclei. Both findings were important, because they indicated success in transfecting the desired cell type and cell compartment, although the underlying mechanism remains largely unknown ((26) for an overview of theories that have been proposed regarding the cellular uptake and intracellular trafficking of AONs), it is important that they are taken up and end up in the nucleus, since splicing takes place in the nucleus. Therefore, for any experimental or therapeutic effect of the AONs, this condition should be met.

Finally, we showed that a single injection of AONs targeting the SRC-1e specific exon in the CeA could result in exon skipping and a shift in expression ratio of the two SRC-1 splice variants in favour of SRC-1a, three and seven days post- injection. The expression ratio shift was not accompanied by differences in the expression levels of total SRC-1, indicating that the effects were selective for the SRC-1e specific exon, leaving total expression levels intact.

Taken together, our results showed that exon skipping may be an appropriate technique for interference with gene expression in the brain, either for experimental or therapeutic purposes. In our hands, it was characterized by specificity for SRC-1e, leaving total SRC-1 expression unaltered, as well as GR and SRC-2 expression, limited immunogenicity and high efficiency. Compared to siRNA methods or the use of viral constructs, it may offer the advantage of not causing cell death, since it does not use any intracellular machinery, thus it limits its interference with normal cellular functions (27, 28).

The fact that AONs were still detectable and active seven days after a single injection may be useful for their applications as experimental tools, as it may be possible to avoid more invasive administration to the brain such as cannulation or repeated administration. Although this may still be necessary for longer experimental designs, for our purposes a single injection was sufficient to establish the desired SRC-1a:SRC-1e expression pattern throughout the experiment (23, 29).

Considering that the majority of genes expressed in the brain undergo alternative splicing AON-mediated exon skipping has high potential (30). If one considers also the use of AON-mediated exon skipping to selectively remove exons with known or unknown functions, thus leading to the expression of truncated proteins or internal deletions, the possibilities become endless. Similarly, alternative splicing may also be relevant for therapeutic interventions, either via splice variant selection or by restoration of the reading frame of mutated pre-mRNA molecules. Obviously, there are many more considerations before moving to human use such as safety, administration and efficacy; however, for some disease models AON-mediated exon skipping has shown very promising results (23, 31-33).

## Functional consequences of SRC-1 isoform switching in the CeA

As the naturally occurring expression pattern of the two SRC-1 splice variants in the CeA favors SRC-1e (34), we sought to investigate what the effects of a shift of their expression ratio in favor of SRC-1a would be on the regulation of CRH expression by glucocorticoids as well as on stress-related behavior and fear memory. The CeA is an important area for the orchestration of appropriate responses to stressors and acquisition and expression of fear conditioning. GR signaling has been shown to be indispensable for those functions, as local knockdown of GR expression in the CeA results in fear conditioning impairments which can be rescued by ICV administration of CRH (35). In addition, GR knockdown in the CeA results in abrogation of CRH expression regulation by glucocorticoids (35). Moreover, it has been shown that SRC-1 expression in the CeA is necessary for proper regulation of CRH expression by glucocorticoids and normal basal CRH expression in the CeA (36). Finally, the two SRC-1 splice variants appear to have different effects on the regulation of the *crh* promoter; SRC-1a represses the *crh* promoter, whereas SRC-1e lacks repressive capacity (37).

To test basal anxiety and consolidation of fear memory, we used two well-described paradigms: the open field and fear conditioning, respectively. Subsequently, we tested the effects on SRC-1 isoform switching on the regulation of CRH expression by glucocorticoids in the CeA. Our results suggested that a shift in expression ratio in favor of SRC-1a in the CeA leads to increased locomotion and impairments in a fear conditioning paradigm, as well as abrogation of CRH mRNA induction by chronic exposure to the synthetic glucocorticoid dexamethasone. These findings underline for the first time *in vivo* the importance of SRC-1 for glucocorticoid signaling, as well as the differential effects of the two SRC-1 splice variants on the *crh* promoter. Interestingly, we found a positive correlation between the SRC-1a:SRC-1e expression ratio and the total distance walked in the open field, which may indicate a direct relationship between the expression ratio of the two splice variants locomotor activity.

The most striking effect was the complete blockade of the dexamethasone-induced CRH expression upregulation in the CeA after the expression ratio shift of the two splice variants. Here, it is important to emphasize the difference between the two SRC-1 splice variants in their affinity for the GR; the SRC-1a-specific NR box has higher affinity for the GR than the three central NR boxes (38). Thus, the effects of SRC-1a in the CeA may be amplified due to its higher affinity for the GR, rather than dependent on simple stoichiometry of the two splice variants.

Another open question regards the cause of the observed behavioral differences. The fear conditioning results could be, at least to some extent, explained by the known effects of the two splice variants on CRH expression (36, 37). Kolber et al., showed that GR-dependent expression of CRH in the CeA is necessary for proper acquisition and consolidation of fear conditioning (35). However, we did not find differences in CRH expression after saline treatment (which are expected to be very close to basal levels), therefore, the differences in open field could not be easily explained in relation to CRH expression and function. Similarly, there were no differences in HPA axis reactivity at basal conditions or after stress. Importantly, basal CRH expression in the CeA may not be dependent on GR at all, as shown

by the modest effects observed after adrenalectomy (39). Considering the mode of action of coregulators, it is plausible that there are more GR-target genes differentially regulated by the two SRC-1 splice variants. To cast light to this issue further research is necessary employing broader gene expression analysis techniques such as mRNA microarrays or RNA sequencing to identify those “elusive” genes. In addition, given the interactions of coregulators with other nuclear receptors, such as the estrogen receptor (40), it would be useful to profile the interactions of the two SRC-1 splice variants with other coregulators or pathways of other transcription factors and nuclear receptors. For example, SRC-1 is known to interact with CBP/p300, a coregulator of CREB (41). CREB plays an important role in the activation of the CRH promoter, therefore, it would be essential to understand the extent of interplay between CREB- and GR-dependent transcriptional pathways and the role of the SRC-1 isoforms therein.

In conclusion, splicing modulation and shifting of the expression ratio of naturally occurring splice variants may be of relevance for brain function. Furthermore, manipulation of downstream components of GR signaling may be of relevance for psychopathology, since they offer higher specificity than, for instance GR antagonism or GR knockdown. Finally, it suggests that SRC-1 and its splice variants may be possible targets for manipulation and of therapeutic relevance for psychopathology.

### **Interactions of liganded GR with coregulators**

There is no comprehensive overview of the coregulators that interact with MR and/or GR. Moreover, for known coregulators, we have often little knowledge about the neuromodulatory actions in which they may be involved. The expression of all putative coregulators for MR and GR is available for both mouse and human in databases such as the Allen Brain Atlas (for a number of examples see: (42)). To interpret the expression data in a meaningful way, it is important to know which of the putative coregulators can interact with the receptors. The approach we used in chapters 4 and 5 to investigate the induced interactions by different ligands between the GR and a set of coregulators was the MARCoNI assay. This assay measures one-to-one binding of a given NR to a set of coregulators. The latter are represented as helical peptides of functional NR-box motifs, or their repressor protein equivalent (CoRNR-box), selected from a broad base of literature. This set (>150) of peptides is immobilized in a micro-array format and NR binding is quantified using fluorescently labeled antibody (43). The NR-coregulator interaction profile serves as a sensor for receptor conformation and thus status of the AF-2 of the receptor (44). Functional modulation, e.g. by ligand, mutation or post-translational modification of NRs, recombinant but also in whole-cell lysates (45) can hence be studied by quantification of coregulator interactions. Since this approach involves the use of only the LBD region, we lack relevant information regarding AF-1 (which may also be ligand independent (46)), interactions with other transcription factors (and transrepression activity mediated by them) (47), as well effects on non-genomic GR signaling (48).

Assays like these will be of great assistance to identify relevant coactivators for individual members of the nuclear receptor superfamily. Combining functional interaction data with

expression data like those in the Allen Brain Atlas may bring us a long way to defining the coregulators that are involved in MR and GR signaling in particular brain regions.

### **Targeting GR with novel GR ligands**

Besides targeting directly the expression or splicing of coregulators, it may be useful to modify the interactions between the GR and the coregulators that are present in a certain cellular context. In this regard, pharmacological modulation of the GR may be of particular interest both in the brain and the periphery. Classically, pharmacological manipulations were restricted to the use of agonists or antagonists. However, this approach has some limitations. The use of antagonists such as RU486, for instance in the treatment of the effects of hypercortisolemia, is characterized by some disadvantages which limit their therapeutic potential. One important issue is selectivity for the GR. RU486 binds also the progesterone receptor, thus acting as an abortifacient. There have been several attempts to design ligands with increased affinity for the GR compared to other receptors (49-51). The second important issue is that total GR antagonism may disinhibit the HPA axis, resulting in the elevation of glucocorticoid levels. In addition, it may not be desirable to block all GR-dependent effects, since some of them are beneficial for proper cognitive and memory functions. Hence, the use of selective GR ligands has been attempted to provide more specific modulation of the GR and block certain pathways while leaving others intact. These include attempts to develop GR ligands that retain their anti-inflammatory properties, without effects on metabolism (52-56).

In chapter 4 we profiled the novel selective GR ligand C108297. We found that it induced a unique GR-coregulator interaction profile, resembling features of both agonists and antagonists. In particular, several GR-coregulator interactions were blocked, however, the SRC-1a specific NR box was preferentially recruited. On the other hand, there was no induction of GR-corepressor interactions. We also found mixed effects on gene expression in the brain with both agonistic and antagonistic effects. Notably, there was no disinhibition of the HPA axis, and we found agonistic effects on inhibitory avoidance but antagonism in the effects of corticosterone on adult neurogenesis. C108297 showed mild suppression of post-stress CRH expression levels in PVN, but lacked any effects in the CeA.

In chapter 5 we studied the effects of a novel GR ligand (C118335) on gene expression in the brain and inhibitory avoidance behavior. This compound induced *in vitro* a GR-coregulator interaction profile which resembled that of an antagonist, with some notable exceptions, such as the preferential recruitment of SRC-1 NR-box IV. Moreover, it was shown to be efficient against olanzapine-induced increase of body weight in rats, suggesting an RU486 like efficacy (57). We found that C118335 antagonized corticosterone-induced gene expression in the brain, and attenuated the consolidation of an inhibitory avoidance test. Interestingly, C118335 did not disinhibit the HPA axis. Taken together, our data suggest that C118335 may be an improved GR antagonist compared to RU486. The two novel ligands that were tested showed distinct molecular interactions in the Marconi assay, which partly explained their *in vivo* efficacy. However, we are not able to predict the pharmacology of the compounds with a single assay, because the receptors can act via at least three distinct action mechanisms that



may be separately targeted. First, non-genomic signalling can take place either via membrane-associated variants of the classical receptors (58, 59), or via cytoplasmic receptors (60). Second, transcriptional signalling can occur in a manner that depends on interaction with other transcription factors. AP-1 and NF- $\kappa$ B are well-known examples, but which interactions bear most relevance for the brain is mostly unknown (61). Thirdly, GR and MR can bind to the DNA in their classical GRE-dependent manner, and subsequently interact with any of tens of other transcription factors and coregulator proteins that constitute the actual signal transduction of the receptors.

MR and GR always mediate hormone actions in a given cellular context – which may affect fear, memory, reward, or other aspects of cognitive and emotional processing, depending on the demands on the organism. The receptors do so via cross-talk with other signalling cascades that are activated, for instance, by glutamatergic or noradrenergic excitatory input. Much of the cross-talk may take place at the level of transcriptional coregulators that are common to the signal transduction of MR/GR and the cAMP-coupled transcription factor CREB (41). Furthermore, cross-talk may also take place at the DNA level, either by one factor pioneering the binding site of another, or by binding to the same coregulator or transcription factor (46, 62).

In order to make progress, basic knowledge of possible coregulators of MR and GR can be combined with the comprehensive expression databases that are available. The first reports on genome-wide DNA targets by ChIP-seq (61, 63) should be complemented with similar profiles of coregulators. However, the outcome of such experiments will depend on the particular context the animal is in (see (64) for an example of liver targets of GR in fed or fasted state). Of course, a better use of available transgenic (knock-in) mouse lines that allow functional dissection of GR (and MR) signalling pathways (such as the GR<sup>dim/dim</sup> (65) or CBP<sup>KIX</sup> mice (66), or mice with altered GR:MR expression ratio (67)) may be used to a larger degree. Lastly, the selective receptor modulators that are already available, and of which the mechanism is understood, may be used to distinguish between different signalling pathways, using straightforward pharmacological approaches. The useful application of existing SGRMs, and the development of novel selective modulators for both MR and GR may not only help to understand how glucocorticoids modulate brain function, but also may be used in future for therapeutic use in stress-related psychopathology. In this regard, our data suggest that C108297 and C118335 may be good candidates.

### **Modulation of nuclear receptor function via targeting of coregulators**

Although the work described here has focused on GR-function, the common mechanism of action of nuclear receptors allows for generalization of the model. Because of the broad expression of these receptors in many cell types and tissues, targeting with classical agonists/antagonists has been often proven suboptimal due to side effects. However, many coregulators show a more specific and limited expression pattern such as SRC-3 in the brain where it is expressed mainly in the hippocampus, cortex and olfactory bulbs and the differential distribution of the (68, 69) splice variants of SRC-1 (34). Moreover, selective recruitment of

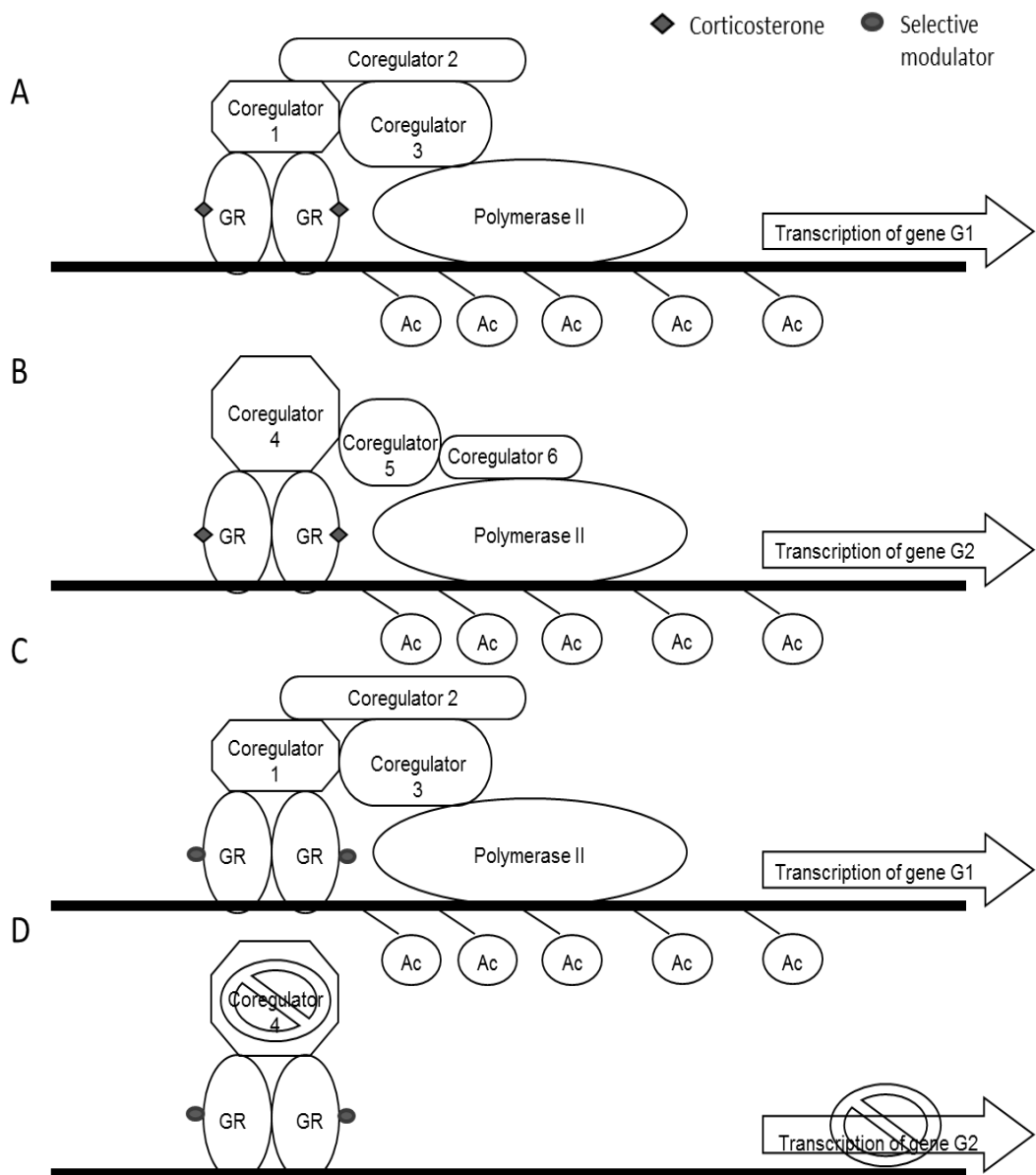


Figure 1. Proposed model of the function of selective modulators. A-B. The glucocorticoid receptor is bound to its natural ligand corticosterone, dimerized and on chromatin. It can recruit a number of different coregulators that interact directly with it (1,4), which can, in turn, recruit other coregulators (2,3,5 and 6). These GR-coregulator complexes can then stabilize the transcriptional machinery, acetylate histones and activate the transcription of genes G1 and G2. C-D. When GR binds a selective modulator it only induces/allows interaction with coregulator 1, but not 4. Therefore, only transcription of G1 takes place, while the transcription of G2 is blocked.

coregulators may change the directionality of the transcriptional effects of nuclear receptors towards the transactivation or transrepression of specific genes. Therefore, the use of ligands that result in specific recruitment of coregulators may be advantageous. An example that illustrates this principle is the use of the GR ligands C108297 and C118335 that show antagonistic effects without disinhibiting the HPA axis.

Alternatively, it is possible to modulate the expression of coregulators locally. Because of the plethora of interactions between coregulators and various nuclear receptors, global deletion of coregulators may not be ideal since it would affect different nuclear receptor-dependent pathways and may induce the development of compensatory mechanisms (36, 70). Even relatively subtle manipulations may have broader effects and this is something that needs to be taken into account for both experimental and therapeutic approaches.

## Conclusions

From the research described here the following conclusions can be drawn:

- Antisense mediated exon skipping is a feasible method to study the function of genes locally in the brain.
- Shifting of the SRC-1a:SRC-1e expression ratio in favour of SRC-1a changes glucocorticoid sensitivity in the CeA, as measured by abrogation of the dexamethasone-induced upregulation of CRH expression in this cell group and the impaired fear-motivated behavior.
- C108297 is a selective modulator of the GR with mixed agonist and antagonist function that can antagonize some of the GR-dependent effects without leading to disinhibition of the HPA axis.
- C118335 is a novel GR ligand with a mainly antagonistic profile antagonizing GR-dependent effects on gene expression in the brain and impaired consolidation of fear memory.
- The approaches described here may offer new possibilities for the targeted modulation of GR-dependent effects in the brain.

## Future perspectives

Despite the work described here, several questions remain unanswered. Future research should be oriented to cast light on the function of the SRC-1 splice variants in response to chronic stress and particularly whether this manipulation in the CeA would result in alterations of HPA function. In addition, since most of the *in vivo* work regarding SRC-1 function has been performed on SRC-1 KO animals which develop well-documented compensatory mechanisms, it would be worthwhile to attempt to interfere with total SRC-1 expression either via virally-mediated knockdown or with the use of AONs. This strategy would permit to investigate the effects of SRC-1 ablation on GR-signaling in the absence of compensatory mechanisms. Another relevant open question is the function of SRC-1 in response to stress in other brain region beyond the CeA.

At a different level there are outstanding questions regarding the gene targets of each splice variant/coregulator and which protein cocktail is recruited to each particular context. There has been success recently in developing ligands that recruit coregulators in a selective and specific manner (71). Therefore, knowledge of coregulator recruitment to the promoters of certain genes may assist the development of ligands that can affect the expression of genes with high specificity depending on cellular context.

Coregulators can be involved in epigenetic regulation of gene expression either via own activity or via recruitment of appropriate proteins. Thus, studying their epigenetic effects in relation to the changes that appear after exposure to stress (72, 73), early life adversity (74) or acquisition, consolidation and recollection of traumatic memories (75) may provide a new level of possibilities for regulation.

Finally, development of new selective GR or MR modulators, and better characterization of the currently available molecules is promising to open new avenues for the successful treatment of stress-related psychopathology.

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