

Spadin, a Sortilin-derived peptide: a new concept in the antidepressant drug design

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Abstract: Depression is the most common of psychiatric illnesses. The design of effective treatments for this disorder is a challenging process. Recently, the two-pore domain potassium channel TREK-1 has been identified as a new target in depression, and its antagonists might become effective antidepressants. Deletion of TREK-1 gene results in a depression-resistant phenotype that mimics antidepressant treatments. Here, we validate the fast antidepressant effects of spadin, a secreted peptide derived from the propeptide generated by the maturation of the sortilin receptor and acting through TREK-1 inhibition.

Key words: Spadin, TREK-1 channel, depression, behavior

Depression is a devastating illness that affects ~17% of the population at some point in life, resulting in major social and economic consequences (Kessler *et al.*, 2005). The use of antidepressants has an overall low clinical efficacy as full remission only occurs in one-third of the patients. In the case of response, side effects are often observed, as well as a delay in the onset of therapeutic efficiency. Most antidepressants increase levels of the monoamine serotonin (5-HT) and/or noradrenaline (NA), suggesting that biochemical imbalances within the 5-HT/NA systems may underlie the pathogenesis of this disorder (Nestler *et al.*, 2002; Krishnan and Nestler, 2008). To date, the mainstay of antidepressant treatments is constituted by selective serotonin reuptake inhibitors, which inhibit the 5-HT reuptake pump. Improving the treatment of depression is challenging. Recently, mouse models of depression have highlighted the putative role of the TREK-1 channel in the mechanisms of action of antidepressants. The TREK-1 channel (*figure 1A*) belongs to the particular family of two-pore-domain potassium channels (Lesage and Lazdunski, 2000). It is open at membrane potentials in

physiological conditions and contributes to the background or leak currents that set the resting potential and oppose depolarizing influences. TREK-1 is activated by volatile general anesthetics, membrane stretch, heat, acidosis, but also by riluzole, polyunsaturated fatty acids and lysophospholipids (Lesage and Lazdunski, 2000; Honore, 2007). These openers prevent neuronal death in animal models of epilepsy, stroke, global, retinal and spinal ischemia (Blondeau *et al.*, 2002a,b; Blondeau *et al.*, 2007; Ettaiche *et al.*, 1999; Heurteaux *et al.*, 2006a; Lang-Lazdunski *et al.*, 1999, 2003; Lauritzen *et al.*, 2000). The phenotyping of TREK1 deficient mice (*kcnk2^{-/-}*) has identified a major role for TREK1 channels in the control of epileptogenesis, general anesthesia, polyunsaturated fatty acids-induced neuroprotection (Heurteaux *et al.*, 2004) and pain (Alloui *et al.*, 2006; Noel *et al.*, 2009). The TREK-1 channel is physiologically blocked by phosphorylation processes such as phosphorylation resulting from Gs or Gq G-proteins. It is also inhibited by SSRIs such as fluoxetine (Heurteaux *et al.*, 2006). Deletion of the TREK-1 gene (also called *kcnk2*) results in a depression-resistant phenotype that mimics treatment with

antidepressants (Heurteaux *et al.*, 2006). Interestingly, the Star*D study has identified an association between the existence of four genetic variants (SNPs) in the TREK-1 locus, and a resistance to multiple antidepressant classes (Perlis *et al.*, 2008; Dillon *et al.*, 2010). All these findings indicate that 1) genetic variations in TREK-1 may identify individuals at risk for depression treatment resistance and 2) a search of selective blockers of TREK-1, hitherto not available, might potentially lead to a new generation of antidepressants.

Growing evidence indicates that trafficking and addressing as well as functional properties of native ion channels depend on their lipidic and proteic environments. K⁺ channels are known to interact with partner proteins that are crucial for their regulation. In search of protein partners, we identified the sortilin receptor (Mazella *et al.*, 1998; Nielsen *et al.*, 2001) in the regulation of the channel function (*figure 1B*). Sortilin is a 95-100-kDa type-1 membrane protein, consisting of a large luminal domain, a single transmembrane segment and a short C-terminal cytoplasmic tail. A large part of sortilin is localized at the level of the

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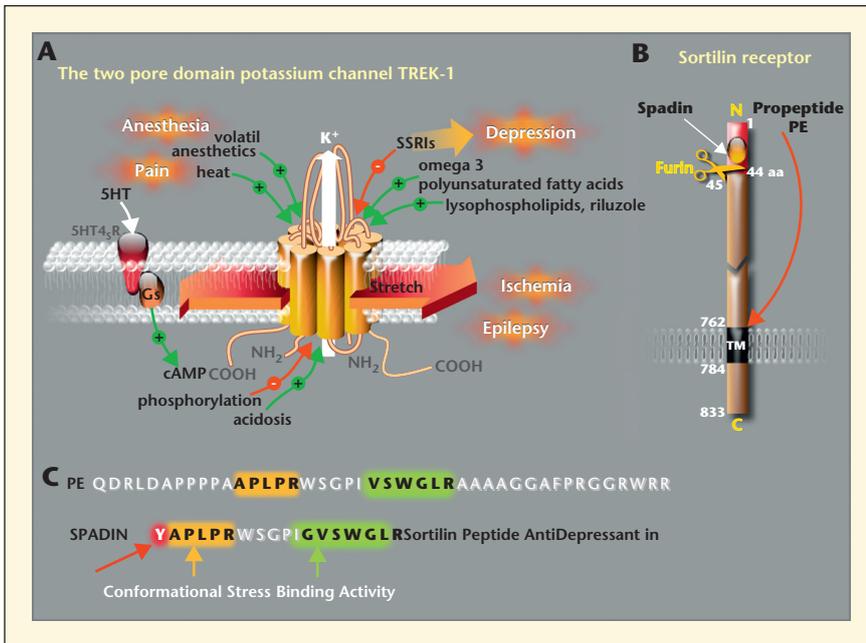


Figure 1. *The two pore domain potassium channel TREK1 and the sortilin receptor :*
 A) Activation and Inhibition of TREK-1 and involvement of TREK-1 in cerebral pathologies ;
 B) Cleavage of the mature ligand-binding receptor by furin and release of the propeptide (PE).
 C) Identification of spadin sequence.

Golgi apparatus where the protein triggers intracellular functions of trafficking. Depending on its cellular location, sortilin may also act as a receptor or a co-receptor and binds neurotensin (NT), the precursor of the Nerve Growth Factor (proNGF), the receptor-associated protein (RAP), the lipoprotein lipase and the propeptide released from its precursor form (Mazella *et al.*, 1998; Nykjaer *et al.*, 2004). Sortilin as well as TREK-1 are highly expressed in cerebral structures involved in the pathophysiology of depression, such as prefrontal and cingulate cortex, amygdala, hippocampus, nucleus accumbens, dorsal raphe and hypothalamus. Sortilin is synthesized as a proform (prosortilin) which, in late Golgi compartments, is converted to the functional ligand-binding receptor by cleavage and release of a 44 residue N-terminal propeptide (Gln¹-Arg⁴⁴, propeptide) by furin (Munck Petersen *et al.*, 1999). Propeptide binds to the mature receptor with a high affinity (K_d ~ 5 nM). Structure-function relationship studies have identified that the peptide Gln¹-Arg²⁸ was as efficient on the binding activity as the entire propeptide Gln¹-Arg⁴⁴, whereas the affinity of the peptide Gln¹-Arg¹⁶ was very low (Westergaard *et al.*, 2004) (figure 1C). Therefore, we designed the peptide spadin by conserving the sequence 17-28 in which we added the sequence 12-16 (APLRP) in order to maintain conformational stress. This partial propeptide (Ala¹²-Arg²⁸) called spadin (figure 1C) was tested for its potential effects on TREK-1 channel regulation and for its validation as antidepressant drug in five behavioral models of depression (Mazella *et al.*, 2010).

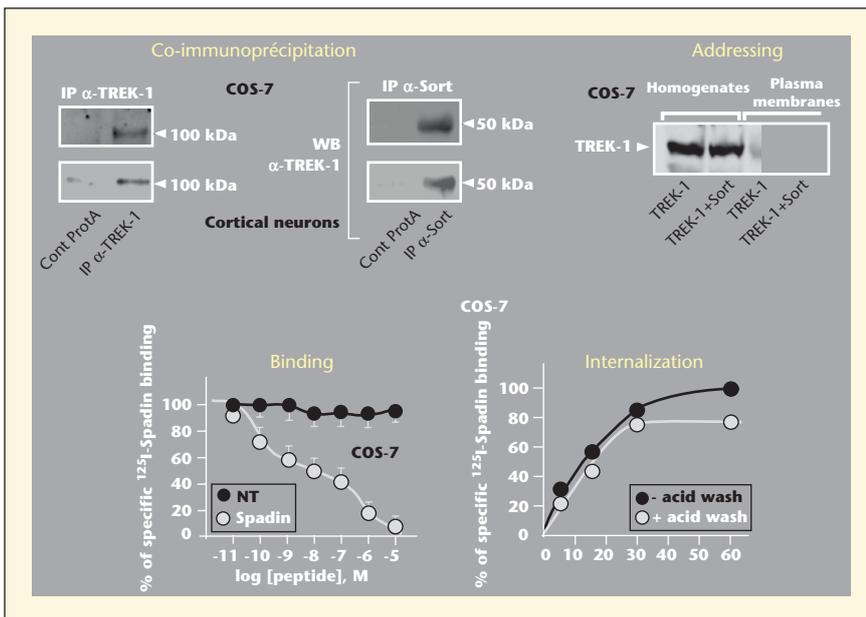


Figure 2. *Sortilin and Spadin interact with the TREK-1 channel. Co-immunoprecipitation: Immunoprecipitation of Sortilin with anti-TREK-1 antibody (IP α-TREK-1) or immunoprecipitation of TREK-1 with anti- Sortilin antibody (IP α-Sort) from transfected COS-7 cells or mouse cortical neurons. Immunoprecipitated proteins are subjected to Western blots and revealed using anti-sortilin (WB: α-Sort) or anti-TREK-1 (WB: α-TREK-1). Addressing: Influence of Sortilin on the expression of TREK-1 at the plasma membranes. COS-7 cells are transfected with TREK-1 in the absence or in the presence of Sortilin. Crude homogenates, purified plasma membrane proteins or cell surface biotinylated proteins are subjected to Western blot analysis and revealed using anti-TREK-1 antibody. Binding: Competition between ¹²⁵I-Spadin and unlabeled Spadin (white circles) or NT (black circles) for binding to TREK-1 transfected COS-7 cell homogenates. Internalization, Association kinetics of ¹²⁵I-Spadin binding to COS-7 cells transfected with TREK-1. At the indicated times, cells are either washed twice with 500 μL of binding buffer (black circles) or treated with 500 μL of acid-NaCl buffer for 2 min (white circles).*

The first result of this work is the identification of the *NTSR3/Sortilin receptor as a novel TREK-1 partner protein* (figure 2). It interacts physically and functionally with TREK-1 to modify its cell surface expression. Experiments of immunoprecipitation of TREK-1 and Sortilin showed that each antibody immunoprecipitated the tested partner, i.e. sortilin precipitated with the TREK-1 antiserum, and TREK-1 with the anti-

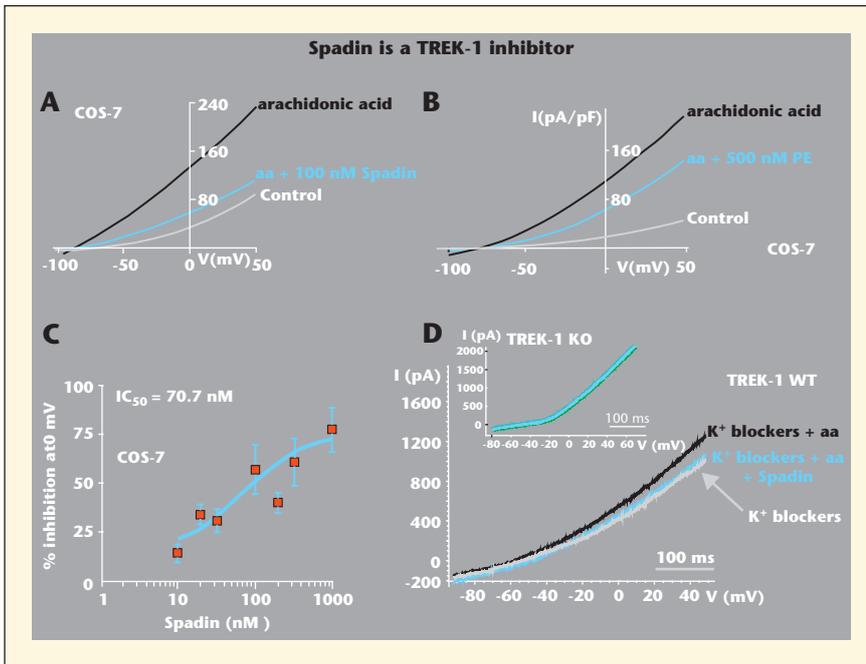


Figure 3. Effects of Spadin on the TREK-1 channel activity. A-B) Whole-cell currents measured in COS-7 transfected cells in presence of potassium blockers (K^+ blockers, 10 mM tetraethyl ammonium (TEA), 3 mM 4-aminopyridine (4-AP), 50 nM charybdotoxin, 10 μ M glibenclamide, 100 nM apamin). Cells are clamped at -80 mV and voltage changes are applied by ramp from -100 to 50 mV, 1 s in duration. Currents are recorded after TREK-1 activation by 10 μ M arachidonic acid (aa) and aa + Spadin (100 nM) (A) or aa + propeptide (PE, 500 nM) (B). Native currents are recorded in the absence (Control) and in the presence of spadin or PE.

C) Dose-dependent spadin inhibition of TREK-1 currents, IC_{50} value at 0 mV is of 70.7 nM. Currents are measured in the presence of 10 μ M aa. D, Native currents recorded in the presence of K^+ blockers after stimulation by 10 μ M of aa on CA3 pyramidal neurons from hippocampus slices in wild-type mice (D) or in *kcnk2* deficient mice (TREK-1 KO) (Inset) in the presence or the absence of spadin (1 μ M). Currents are elicited by a ramp from -100 mV to 50 mV.

sortilin antibody (figure 2 Co-immunoprecipitation), in both COS-7 cells and cortical neurons co-expressing both proteins. The expression of TREK-1 within the plasma membranes, measured either by preparing purified plasma membranes or by using cell surface biotinylation, was enhanced (by a factor 3 and 6, respectively) when COS-7 cells were cotransfected with sortilin (figure 2 Addressing), confirming the interaction between the two proteins, at least during the channel sorting. Spadin displays identical binding and functional properties as those of the full length propeptide on the neurotensin system. Competition experiments between 125 I-labelled spadin and unlabelled spadin or NT on membrane homogenates from TREK-1 transfected COS-7 cells showed that spadin bound specifically to TREK-1 with an affinity of about 10 nM. This binding was selective

since NT was unable to displace the binding of 125 I-spadin (figure 2 Binding). Association kinetics of 125 I-labelled spadin on whole COS-7 cells expressing TREK-1 showed that the radioactivity associated with cells reached a plateau within 30 min. Removal of surface-bound radioactivity by acid-NaCl wash revealed that about 80% of total 125 I-labelled spadin bound at this time was intracellular, indicating that spadin was internalized with TREK-1 following interaction (figure 2 Internalization). These data strongly suggest that sortilin constitutes a sorting partner of the TREK-1 channel.

Electrophysiological studies identify a new function for spadin as a peptidic antagonist of the TREK-1 channel. As previously described (Honore, 2007), TREK-1 basal channel activity was strongly and reversibly activated by

arachidonic acid (aa, 10 μ M), which induced a typical TREK-1 background current, characterized by outward rectification reversed at the predicted value for E_{K^+} . Using the whole-cell patch-clamp technique on TREK-1 transfected COS-7 cells, we demonstrated that 100 nM of spadin were able to block $63 \pm 12\%$ of the TREK-1 current stimulated by aa (figure 3A). A spadin dose-response experiment indicated an IC_{50} value of 70.7 nM at 0 mV (figure 3C). Spadin displayed a better affinity than the propeptide, since 500 nM of propeptide were necessary to block $41 \pm 5\%$ of the aa stimulated TREK-1 current measured at 0 mV (figure 3B). Spadin also blocked the TREK-1 activity in CA3 hippocampal neurons on brain slices of wild-type mice and not in *kcnk2*^{-/-} mice, suggesting a specific inhibitory effect of spadin on the TREK-1 channel (figure 3D).

Finally, we point out spadin as the first peptidic and fast-acting antidepressant. Spadin efficacy was first assessed in the forced swimming test (FST), which is a highly reliable predictor for antidepressant potential (Krishnan and Nestler, 2008). Spadin was administered 30 min before the test by intracerebroventricular (i.c.v.), intravenous (i.v.) or intraperitoneal (i.p.) route at doses of 10^{-4} to 10^{-8} M. When placed in an inescapable cylinder of water, spadin-treated mice exhibited reduced floating or immobility times in the three modes of injection with respect to their saline-treated counterparts (figure 4A). The immobility is interpreted as "a state of despair". The dose-responses of spadin showed that the highest reduced immobility times were observed at the dose of 10^{-7} M in i.c.v. (66.8%), 10^{-6} M in i.v. (62.9%) and 10^{-5} M in i.p. (55.30%) administration. The magnitude of the antidepressant behavior was similar to that observed in fluoxetine-treated wild-type and saline-injected *kcnk2*^{-/-} mice. It has been proposed that a direct facilitation of 5-HT firing rate in the dorsal raphe nucleus (DRN) should be a requirement for a faster onset of antidepressant action (Blier, 2001). Interestingly, in *kcnk2*^{-/-} mice we observed an increase in the firing activity of DRN 5-HT neurons (Heurteaux et al., 2006). Obviously, such results raise the possibility that spadin could exert a rapid onset of action. A 4-day treatment with spadin (i.v., 10^{-6} M) significantly reduced the time spent immobile by

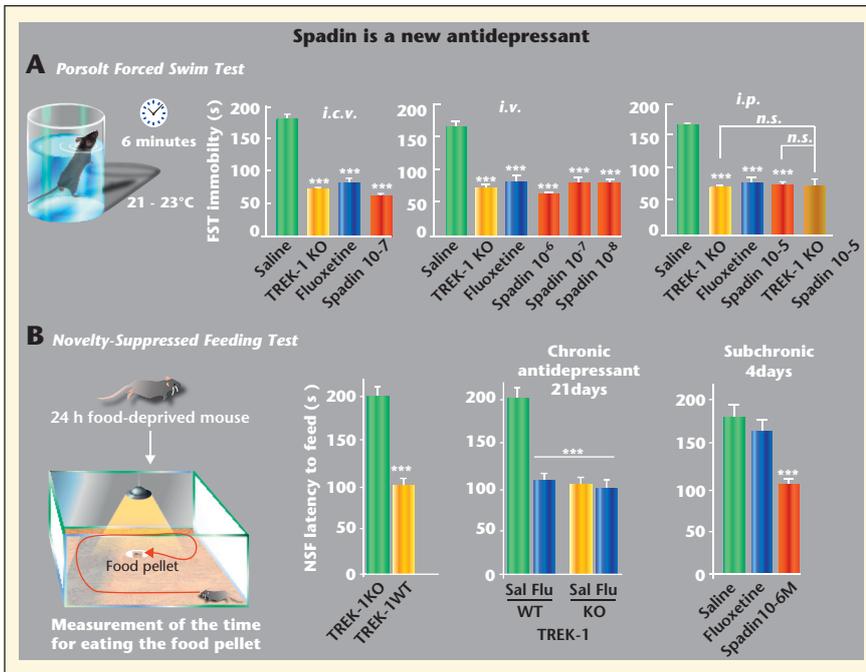


Figure 4. Antidepressant effects of Spadin. A) Forced Swimming Test (FST), spadin-treated mice have a shorter time of immobility comparable to that obtained with TREK-1 KO or fluoxetine-treated mice, whatever the way of spadin administration: intracerebroventricular (i.c.v.), intravenous (i.v) or intraperitoneal (i.p). Acute treatments: Spadin (10^{-4} to 10^{-8} M) or Fluoxetine (3 mg/kg) or Saline solutions are injected 30 min before the test in wild-type and TREK-1 KO mice. B) Novelty-Suppressed Feeding Test (NSF): Subchronic treatments: Spadin (10^{-6} M), Fluoxetine (1 mg/kg) or Saline solutions are i.v. injected in a 100 μ L bolus once a day for 4 successive days before the test. In chronic treatments, spadin (10^{-6} M) and fluoxetine (1 mg/kg) are i.v. injected in a 100 μ L bolus once a day for 21 successive days. At the end of the AD treatment, animals are food deprived for one day and then measured for their latency to feed. 4-day treatment with Spadin significantly reduces the latency to feed when compared to saline or fluoxetine treatments.

43.2% in FST (data not shown). In contrast, subchronic fluoxetine treatment had no effect when compared with saline (data not shown). The Novelty Suppressed Feeding test (NSF) is usually carried out for demonstrating antidepressant efficacy after chronic, but not acute treatment (Santarelli *et al.*, 2003). Mice treated with spadin (i.v., 10^{-6} M) for 4 days showed a significant decrease in latency to feed relative to saline-injected animals (figure 4B). A 4-day regimen with fluoxetine (i.p., 3 mg/kg) had no effect in the same conditions (figure 4B). In comparison, a chronic antidepressant treatment with fluoxetine during 21 days in wild-type and *kcnk2*^{-/-} mice significantly reduced the latency to feed as compared to saline (figure 4B).

The fast-acting antidepressant potential of spadin, observed in FST tests is further

confirmed by its ability to activate the transcription factor cAMP response element-binding protein (CREB) and neurogenesis in the adult mouse hippocampus after a subchronic treatment. Indeed, CREB activity and neurogenesis are considered as specific markers of antidepressant action (Nibuya *et al.*, 1996; Santarelli *et al.*, 2003), but have never been observed before 2 weeks of treatment when using classical antidepressants such as SSRIs. Here, we showed that a 4-day chronic treatment with spadin is able to enhance the pCREB/CREB ratio and consequently increases cell division and proliferation in the SGZ (figures 5A-B). Neurogenesis was analyzed by counting the number of progenitor cells that incorporate the DNA synthesis marker 5-bromo-2'-deoxyuridine (BrdU) and that are differentiated into mature neurons.

Interestingly, a 4-day treatment with spadin (i.p. 10^{-5} M) significantly increased by two-fold the number of BrdU-positive cells with respect to saline conditions (figure 5A). In contrast, a 4-day regimen with fluoxetine had no effect on neurogenesis, but fluoxetine induced a significant increase in the number of BrdU-positive cells when it was administered during 15 days (data not shown). Dual labeling of BrdU and doublecortin (DCX), a specific marker of neuronal precursors revealed that 85.2% of BrdU-labelled cells expressed DCX (data not shown).

As described in *kcnk2*^{-/-} mice (Heurteaux *et al.*, 2006), spadin leads to an *in vivo* increase in efficacy of 5-HT neurotransmission as evidenced by an increased firing activity of DRN 5-HT neurons (figure 5C). The facilitation of central 5-HT transmission constitutes the common property of all the antidepressant strategies. From a mechanical point of view, 5-HT_{1A} autoreceptor stimulation reduces DRN 5-HT neuronal firing and, consequently, 5-HT neurotransmission (Blier and de Montigny, 1999). Inhibition of adenylate cyclase and activation of G-protein-coupled inwardly rectifying K⁺ channels (GIRK) are involved in this negative feedback. The decrease in cAMP concentration (as a result of reduced adenylate cyclase activity) in 5-HT neurons is also thought to induce TREK-1 opening because of a consequent reduction of phosphorylation of Ser333 by PKA (Maingret *et al.*, 2000). According to this model, spadin would induce a depolarization by closing TREK-1 channels and, as described for TREK-1 deficient mice would therefore reduce the negative feedback on 5-HT neurons, resulting in increased 5-HT neurotransmission and in turn in antidepressant-like effects. Direct inhibition of TREK-1 by spadin may also contribute to enhanced 5-HT neuron excitability. Because (i) sortilin is the partner protein of the TREK-1 channel and (ii) both proteins are colocalized in 5-HT-enriched areas known to be involved in the pathophysiology of depression, one may infer that spadin acts predominantly through a modulation of the brain 5-HT circuitry. Nevertheless, we cannot exclude that it can also involve other neurotransmission systems. Whatever the effector pathways involved, the fact that spadin has no effects on *kcnk2*^{-/-} mice indicates that

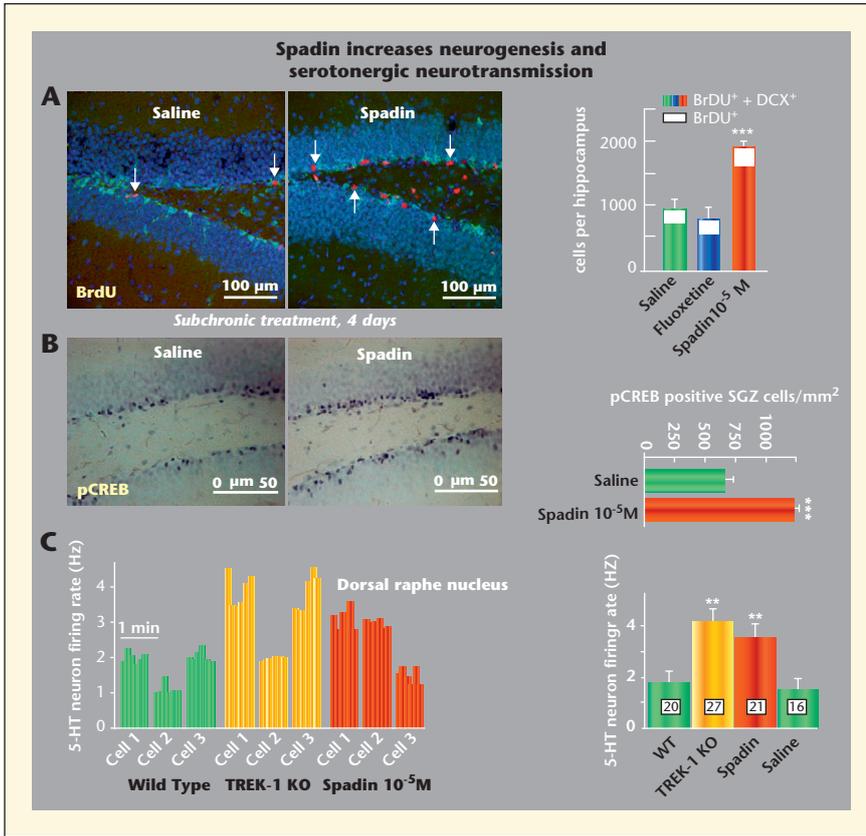


Figure 5. Effects of Spadin on neurogenesis, CREB activation and Serotonergic neurotransmission. A) Spadin increases neurogenesis: (left), Representative photomicrographs of BrdU-labeled neurons in the dentate gyrus of the mouse hippocampus treated either with saline or with spadin (i.v., 10^{-6} M) for 4 days. (Right) Quantitation of BrdU positive cells of hippocampus treated with saline, fluoxetine or spadin (10^{-5} M) for 4 days. 85% of BrdU-labeled cells are positive to DCX. B) Enhanced spadin treatment-induced CREB activation in the hippocampus, as assessed by measuring phosphoCREB (pCREB) immunoreactivity. (left), Immunological distribution of pCREB in the mouse hippocampus after a 4-day i.v. treatment. pCREB is phosphorylated in the cells near the subgranular zone (SGZ). Right: Quantification of pCREB positive cells/mm² in hippocampal SGZ. C) Effect of spadin on the firing rate of DRN 5-HT neurons. Spadin (10^{-5} M in a 100 μ L bolus) or its vehicle is i.p. administered. Recordings start 30 min after the injection, and are performed for a maximal duration of 210 min thereafter. Left: Samples of "descents" performed along the DRN, showing typical integrated firing rate histograms in a vehicle-, TREK-1 KO- or spadin-treated animal. Right: 5-HT neuron firing activity, calculated on the basis of all the cells recorded within the successive tracks performed along the DRN.

its action is first and foremost mediated by a modulation of TREK-1 channels.

Conclusion

Spadin can be considered as a natural endogenous antidepressant and constitutes the first peptide identified as an antidepressant with a rapid onset of action. Due to these peculiar properties, spadin brings a new concept to address the treatment of depression. To date, spadin is also the first blocker

of TREK-1 channel identified, which is not only of relevance in the field of depression, but also constitutes a useful tool to further understand the role of TREK-1 channels in other neurological pathologies. Because it is possible to dose spadin in the serum of depressed patients spadin could become a marker of depression. Detecting and preventing depression certainly could decrease the economic burden of this disease, which is estimated to be 53 billion dollars per year in the United States.

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