Fear memories in Tg*NTRK3* mice, a model of panic disorder: definition of mechanism and search for new therapeutic targets

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TESI DOCTORAL UPF / ANY 2013

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DEPARTMENT CEXS UPF – PHD PROGRAMME IN BIOMEDICINE

I frutti piú belli della scienza della vita, i figli.

Al mio piccolo Carlo.

Ringraziamenti

A long, difficult and intense experience like a PhD is only achievable with the help of many people. Luckily in my case I found plenty of persons who were always on my side, advising me, helping me and enjoying with me my PhD life.

Mara, tu entusiasmo científico ha sido un motor en mi PhD. Gracias por haber creído en mí y haberme concedido la posibilidad de entrar en tu grupo. Gracias por haber invertido dinero y tiempo en escucharme y dejarme realizar mis ideas. Gracias por todos los consejos no solo científicos, sino también de la vida que siempre me has dado. Gracias por haberme machacado en todas mis presentaciones, mis escrituras, mis discusiones… sin duda todo eso ha representado un importante "challenge" (esta palabra que tanto ha representado mi trabajo) en mi experiencia.

Monica, mi ejemplo, mi guru, mi mejor guía científica, la más crítica, la que más me ha hecho trabajar, pensar, discutir…la que me ha enseñado a dudar de mis mismo resultados…en fin una súper dirección de mi tesis. Gracias por haberme enseñado a hacer ciencia, gracias por haberme enseñado a vivir la ciencia, gracias por tu ejemplo. Sin ti seguramente esto no hubiera sido igual…

Oliver, I thank you because you have been a great and always available advisor. Thank you for accepting me in your lab, welcoming me as part of your group and helping me in understanding my work, probably in the most critical moment of it. And thanks for diving with me… Thanks to prof. Linke for teaching me classical mouse surgery, it really helped me. And thanks to all the people met in Magdeburg.

Ignasi, probablemente la persona más disponible y generosa que he encontrado durante esta experiencia. Siempre listo en ayudarme, resolver mis dudas técnicas y científicas, siempre atento a las emociones de la gente y siempre capaz de sacar unas palabras de consuelo. Tu presencia en el lab era fuente de tranquilidad, felicidad y alegría…y por no dejar nunca esta fuente me quedaré a tu lado en nuestra nueva experiencia, ZeClinics.

Marieta, la más experta, el punto de referencia, siempre presente y siempre cariñosa…cualquier duda o pregunta Maria tiene la respuesta, y si no la tiene, la busca y te contesta. Eres muy amable y gran científica…y ahora también una grande madre…Tus sonrisas son capaces de resolver cualquiera de los días malos…gracias por estas sonrisas…y gracias por dedicar tu día de la Mercé para revisar mi tesis. Gracias.

Carmen-cita, sei forte e piena di vitalitá, apprezzo molto il tuo modo di vedere la vita, la tua voglia di conoscere, parlare in tutte le lingue...grazie per avermi accompagnato ed insegnato la magia della neuro-chirurgia...grazie per aver portato uno spirito lottatore come esempio nella mia carriera.

Meritxell (o Merixtell)...es que nunca me acuerdo como se escribe tu nombre, jeje. Sei stata la piú vicina compagna di viaggio in quest'esperienza, grazie per aver condiviso con me ogni momento (triste e felice) di questo viaggio…entrata al lab, congresso a Benasque, PhD symposium e scrittura della tesi…abbiamo condiviso tanto e ti sono molto grato per avermi supportato (e sopportato) dalla stessa barca...Cmq non ti perdoneró mai di non essere venuta al mio matrimonio!!!

Susanita, que fuerza que tienes! Me encanta tu gana de luchar…eres la capitana de verdad…gracias a ti Pepeta se quedará siempre en mi corazón…gracias por encontrar siempre el tiempo de discutir mis resultados, criticarlos y aconsejarme sobre los pasos para conseguir este título. Gracias por las birritas, las calcotadas, las salidas por Barna…gracias!

Thomas, aunque estas lejos (el del final del pasillo) no me olvidaré de ti, padre como yo, buen amigo y experto en physio…gracias por haber compartido conmigo tus conocimientos.

Tizi, un aiuto fondamentale nella realizzazione di questo lavoro. Una grande amica, con la quale potersi confidare, chiacchierare (e sparlare)...grazie per essere sempre attenta e disponibile ad aiutarmi e confortarmi nei momenti tristi. Buona fortuna con la "nuova" vita...

My dear former compañeros del lab, Xavi, Carlota, Gari, Nuno, Ornella y los demas... cadauno de vosotros me dejó algo, nunca me olvidaré y gracias por vuestras ayudas.

A los nuevos del lab, Silvi, Marta, Jose Antonio, Linus animo con el PhD…aprovechad a lo máximo esta experiencia, pasadlo bien y sacar lo máximo que podáis…al final es una buena experiencia (solo al final jeje)…Belén, animo! Me gustaría quedarme a tu lado, pero ya se acabó, tengo que irme…suerte!!!

PhD people, a special thanks to Imma, Aljoscha, Martin, thanks for having shared with me this amazing experience. Aljoscha ha sido un placer viajar contigo.

Italian family, Giuly, Matteo, Luca, Aureola, Umbé, Rubbo, Sere, Jose, Chiara, Silvi e Luis e tutti gli altri grazie per le cene, le serate, le uscitine...sempre un piacere vivere da emigrato, se si incontra bella gente del nostro Bel Paese con la quale condividere la vita.

Paco, Javi, Alex, Mariano, Andrea la compañía de las cervezas, siempre la penúltima! Gracias my friends…!

Romen, Maria, gracias por haberme bienvenido en Barna, por haberme tratado como vuestro peque. No me olvidaré de vuestra ayuda.

Ai miei cari compari, Simone e Valery…chi l'avrebbe mai detto che finivamo assieme? Dalle serate a villa serena, a quelle al plataforma, ai matrimoni, ai fligli, alle societá...mah forse ci vogliamo davvero bene e siamo davvero dei grandi amici...hihihi NON NE HO ALCUN DUBBIO!!! Inutile spendere altre parole per voi, tanto siete insensibili...nada vi voglio trsopppo bbene!!!

Ai miei cari genitori, sorella e la sua bellissima famiglia, grazie grazie grazie! Grazie per aver sempre creduto in me, avermi accompagnato in tutti i passi della mia vita fino ad oggi, avermi appoggiato nelle scelte di uscire, partire, andare per scoprire e conoscere....semplicempente grazie per amarmi.

E per ultimi i piú importanti, i miei TESORI! Grazie mio piccolo Carlo, a te dedico questo lavoro, a te dedicheró la mia intera vita, il tuo arrivo ha sconvolta la mia tesi, ha portato un carico di gioia e felicitá, con nuove responsabilitá... Mia dolce e preziosa metá, cara Egi, grazie per avermi supportato e accompagnato in questa esperienza, senza dubbio tu sei la persona che piú da vicino ha condiviso con me le gioie e i dolori di questo PhD, mi hai sempre confortato e sopportato nei momenti di stress e difficoltá...grazie...grazie per accompagnarmi nella vita, grazie per crescere con me e per far crescere il nostro piccolo...grazie TA.

Davide D'Amico has been funded by a fellowship "La Caixa international PhD program in Biomedicine" at the Center for Genomic Regulation (CRG), Barcelona, Spain, during the entire period of work (2009-2013).

The laboratory is supported by Departament d'Universitats, Recerca i Societat de la Informació (Grups consolidats 09 2009SGR1313). This work was supported by Grants SAF2010-16427, SAF2007-31093-E, and FIS (PI 082038); Marató TV3; the Jerome Lejeune, Koplowitz, and Areces Foundations; and the European Union (LSHG-CT- 2006-037627; CureFXS ERare-EU/FIS PS09102673).

Abstract

Abstract

The neurotrophin tyrosine kinase receptor type 3 (*NTRK3*) gene has been proposed to contribute to the pathological phenotype of panic disorder (PAND), an anxiety disorder characterized by perturbed and exaggerated fear.

In this thesis we hypothesized that PAND could be related to abnormal associative fear learning processes, underlined by a deregulated functioning of the hippocampus – amygdala – medial prefrontal cortex fear circuit. We addressed this hypothesis, by using the unique validated genetic mouse model of PAND, the Tg*NTRK3*, overexpressing the human *NTRK3* gene, encoding for TrkC.

We found that overexpression of *NTRK3* in mice leads to: i) enhanced and extinction resistant hippocampal-dependent fear memories; ii) an aberrant activation of the fear circuit during learning and storage of fear-related information. The underlying mechanisms are possibly related to hippocampal overexcitability.

Our study confirmed the role of the *NTRK3* gene on pathological fear memories and suggests potential effective therapeutical strategies targeting the excitatory/ inhibitory system and the NT3-TrkC pathway to block exaggerated fear in PAND.

Resumen

Estudios genéticos en pacientes sugieren que el gen del receptor tirosina quinasa de la neurotrofina de tipo 3 (*NTRK3*) podría contribuir en la patología del trastorno de pánico (PAND), un trastorno de ansiedad caracterizado por alteración y exageración del miedo.

En esta tesis, proponemos que PAND podría estar relacionado con procesos anormales de aprendizaje asociativo, subyacentes a una alteración en la regulación del funcionamiento del circuito del miedo hipocampo - amígdala – corteza prefrontal media. Hemos estudiado esta hipótesis utilizando el único modelo genético de ratón validado de PAND, el Tg*NTRK3*, que sobreexpresa el

gen humano *NTRK3* codificante para el receptor TrkC. Hemos demostrado que la sobreexpresión de *NTRK3* en ratones produce: i) un incremento en la memoria de miedo dependiente de hipocampo que es además resistente a extinción; ii) la activación alterada del circuito del miedo durante el aprendizaje y almacenamiento de experiencias emocionales intensas negativas. Nuestros resultados sugieren que los mecanismos responsables están relacionados con una sobre excitabilidad en el hipocampo. Nuestro estudio confirma el papel del gen *NTRK3* en la memoria de miedo patológico y sugiere posibles estrategias terapéuticas para bloquear el miedo exagerado en PAND dirigidas al sistema de excitación / inhibición y a la vía de señalización NT3-TrkC.

Presentation

Presentation

In this doctoral thesis we have focused on elucidating the cognitive mechanisms underlying psychopathological fear in one of the most common and debilitating anxiety disorders, panic disorder (PAND). The human *NTRK3* gene, encoding for the neurotrophin-3 (NT3) high affinity receptor, TrkC, has been proposed as a strong candidate (Gratacos, Nadal et al. 2001; Armengol, Gratacos et al. 2002; Muinos-Gimeno, Guidi et al. 2009) to explain PAND susceptibility. Although several pharmacological and cognitive treatments have been used in clinics for PAND patients, none of them seems to be a final cure for the disorder, probably because multiple levels of complexity might contribute to specific symptoms and phases of the disorder. Several years ago we generated a transgenic mouse model of PAND, the Tg*NTRK3* overexpressing the human *NTRK3* gene. Tg*NTRK3* mice recapitulate several signs typical of PAND patients, at behavioural, molecular/cellular and pharmacological levels (Dierssen, Gratacos et al. 2006; Sahun, Delgado-Garcia et al. 2007; Sahun, Gallego et al. 2007; Amador-Arjona, Delgado-Morales et al. 2010; Gallego, Murtra et al. 2010), proofing its face, predictive and construct validity.

This thesis has been devoted to the elucidation of a central problem in PAND, the formation and maintenance of exaggerated fear memories. Specifically we dissected which cognitive domains related to fear memories are affected in Tg*NTRK3* mice and the molecular and neural players involved.

Our experimental approach covered a multidisciplinary level of research, ranging from behavioural neuroscience, molecular and cell biology, and *in vivo* pharmacology. During this work I had the opportunity of interacting with local and international experts of the field and of visiting the laboratory of Prof. Oliver Stork at the Otto-von-Guericke University, Magdeburg, Germany. During my internship there, and under the co-supervision of Prof. Rudiger Linke (Institute of Anatomy), I learned the technical aspects to characterize long range projecting GABAergic hippocampal neurons by performing intra-hippocampus and intra-amygdala stereotaxic guided injections of neuronal tracers. The knowledge acquired during this internship, allowed me to apply intra-cerebral

pharmacological injections in this thesis, which have contributed to identify disturbed local mechanisms in specific brain regions of Tg*NTRK3* mice.

Part of this work has already been published at the Journal of Neuroscience. There are also other articles in preparation (see annex III) that will show for the first time the involvement of the NT3-TrkC system in physiological fear extinction and pathological impaired extinction in Tg*NTRK3* mouse model of PAND. Finally, we are also working on a review article to submit at the Genes Brain and Behaviour journal, regarding our cognitive hypothesis in human PAND pathophysiology. My work has also been presented at several national and international meetings, such as the Spanish Cajal Winter conference, 2009, EMBL Workshops and Symposium, 2009 and 2010, at the 8th FENS forum of European Neuroscience, 2012 and at the 42nd SFN's annual meeting, 2012.

Publications

Santos M *, **D'Amico D ***, Spadoni O, Amador-Arjona A, Stork O, Dierssen M. Hippocampal hyperexcitability underlies enhanced fear memories in Tg*NTRK3*, a panic disorder mouse model. J Neurosci 33(38): 15259-15271. * Both authors equally contributed to this work.

Santos M, **D'Amico D**, Dierssen M. Panic disorder: insights from human neuroimaging and genetic studies and the contribution of animal models. Review article in preparation (peer reviewed invitation by Genes Brain & Behavior).

D'Amico D *, Santos M *, Dierssen M. NT3 induced extinction of fear memories in Tg*NTRK3*, a panic disorder mouse model. Manuscript In preparation. * Both authors equally contributed to this work.

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INTRODUCTION

1. INTRODUCTION

Fear is an intrinsic emotion adopted by most living beings for survival. It is a highly conserved manifestation to both external and internal threatening stimuli, resulting in a coordinated activation of behavioural, autonomic and endocrinal systems, to generate a “fear response”.
Multiple types of fear have been described in many years of animal and human research, and a first important classification distinguish fear responses into innate and learned (LeDoux 2012). The first relies on highly conserved brain circuitry along evolution and is the response to real threatening stimuli even if never encountered previously in life; this is the case of fear to predators (Blanchard and Blanchard 1972; Blanchard, Blanchard et al. 1990), fear to environmental situations that are highly probable to cause hurt or damage (Walker and Davis 2002) and fear to aggressive faces of conspecifics (Adolphs 2008; Davis, Somerville et al. 2011). Learned fear responses consider all types of fear that result from associative learning processes. Here, the co-occurrence of aversive experiences or stimuli with a neutral stimulus will finally result in the formation of a memory that activates fear in every future encounter with the now associated neutral stimulus. Because of its nature, learned fear is the result of a much more complex circuitry combining both brain cognitive and emotional domains (Blanchard and Blanchard 1969; Gerlai 1998).

PAND is an anxiety disorder characterized by frequent panic attacks, defined as intense fear responses with cognitive and autonomic symptoms (DSM-V 2013). A central aspect in this psychiatric condition is that patients develop strong and persistent fear memories often associated to “neutral” environments in which a panic attack was previously experienced (Lissek, Powers et al. 2005).

1.1 Fear memories in animal models

Classically, fear memories in rodents have been studied and classified by the use of a behavioural paradigm defined as conditioned fear; a form of associative learning in which an initially neutral conditioned stimulus (CS,

usually represented by a tone or a light) is associated with an aversive unconditioned stimulus (US, normally a mild foot shock) to generate a conditioned response (Pavlov 1927; Blanchard and Blanchard 1969; Gerlai 1998). In general, the different fear-conditioning paradigms rely on a cognitive process of associative learning and memory, but each one highlighting different neural sub-circuits and cognitive-emotional domains. We here describe briefly the different types of fear memories characterized in rodents, with a special focus on those that may find a relevant endophenotypic translation in PAND.

One of the first described forms of fear memory is the *cue fear memory*, an associative learning process induced by pairing a neutral CS with an aversive US (Johansen, Cain et al. 2011). Upon successful consolidation, a subsequent presentation of the CS is sufficient to induce a strong fear response in animals, recalling the unpleasant US, such as freezing (absence of any movement except for respiration) or startle (jumping) behaviours (Maren and Quirk 2004; Fanselow and Poulos 2005).

A second type is the *temporal fear memory* that is experimentally induced by separating the above described CS and US with a time interval, a trace. In the trace fear conditioning, a temporal dependency of the CS-US is generated and a less predictable outcome of the aversive US during later expositions to the CS is expected (McEchron, Bouwmeester et al. 1998; Huerta, Sun et al. 2000).

Another type of fear memory has been defined as *contextual fear memory*, in which the CS is a specific context, the environment in which the animals receive the aversive US. This generates an associative memory causing the typical fear response of freezing in animals when exposed to the same context (Maren and Holt 2000; Anagnostaras, Gale et al. 2001; Maren, Phan et al. 2013).

The fear discrimination is experimentally induced in animals by exposing them to two different CS that will acquire different valence. One CS associates with the aversive US (CS+) and the other CS not (CS-) (Pearce 1994; Antunes and Moita 2010). Trained animals are able to discriminate the two CS and typically fear to the CS+ and not to CS-. This has also been extrapolated to contextual fear memories where the same principle applies. Here animals are tested for their ability to discriminate two similar contexts, one associated with the US

(CTX+) and the other not (CTX-) (Frankland, Cestari et al. 1998; Antoniadis and McDonald 1999; Fanselow 2010).

Although different types of fear memories have been identified, all rely on common associative learning phases along the learning process (Table 1).

Table 1: Fear learning and memory phases.

Phase	Stimuli	Duration	Behavioural response
Acquisition	CS-US	Minutes	Increasing freezing
Consolidation	None	Hours	Resting
Retrieval	CS	Seconds	High freezing
Extinction	Multiple CS	Hours/ days	Decreasing/ low freezing
Reinstatement	US	Seconds	High freezing
Generalization	Neutral CTX	Seconds/ minutes	Low freezing

Common phases to all types of fear memories can be identified. Briefly, these are summarized as acquisition, when animals are simultaneously exposed to the CS and US; consolidation, occurring in a resting period during which memory is generated; retrieval, when memory is recalled by exposition to the CS; extinction, a new learning process induced by repeated exposures to the CS without reinforcement by the US, resulting in a dissociation and inhibition of the initial fear memory; reinstatement, a re-activation of the extinguished fear memory upon a single re-exposure to the US; generalization, exhibition of the fear response in different contexts and/or environments that were never paired with the original US. CS, conditioned stimulus; US, unconditioned stimulus; CTX, context.

The processes described above depend on different brain structures of the fear circuit and requires adaptive activation patterns of specific cell populations (Bindra 1969; LeDoux 2000; Maren and Quirk 2004). In this respect, knowledge about the different phases of fear processing and how they are affected in particular psychiatric conditions, such as PAND, can be of relevance for clinical translation.

All these different forms of fear memories help in experimental animal studies to understand which specific cognitive-emotional domain could be altered and set up the basis to analyze the cellular and molecular mechanisms involved.

1.2 The fear circuit

A large collection of studies over the last 50 years has dissected the neural circuit underlying fear in many of its phases and types, with a focus in those brain areas involved in the acquisition, retrieval and extinction of Pavlovian associative fear memory.

It is widely accepted that mainly three structures represent the minimal circuit and these are the hippocampus (HP), required for associative memory including encoding, consolidation and retrieval, the amygdala (Amy), the core area in which fear responses are gated and the medial prefrontal cortex (mPFC) which operates an inhibitory modulation of fear. However, several different sub-regions, nuclei and cell types have been described in these brain areas and specific studies have tried to dissect their function (Figure 1, adapted from Sierra-Mercado, Padilla-Coreano et al. 2011).

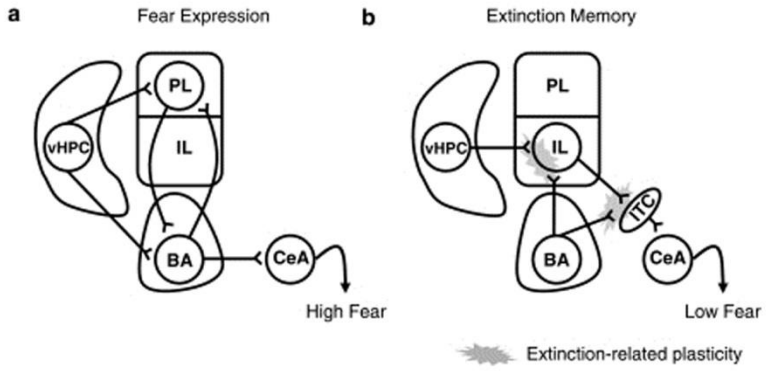

Figure 1: Simplified brain circuit involved in fear expression and extinction. (a) The expression of fear memory relies on inputs from the vHPC to the amygdala and PL region of the mPFC and from the PL directly to the BA amygdala. BA amygdala neurons then activate CeA neurons, which lastly generate high fear responses. (b) When extinction occurs, vHPC inputs to the IL induce extinction-related plasticity mechanisms, resulting in the IL-mediated activation of amygdala ITC, which lastly block CeA neurons to reduce fear responses. vHPC, ventral hippocampus; PL, prelimbic; IL, infralimbic; mPFC, medial prefrontal cortex; BA, basal

amygdala; CeA, central amygdala; ITC, intercalated cell cluster. Adapted from (Sierra-Mercado, Padilla-Coreano et al. 2011).

The hippocampus

The hippocampus (HP) is a heterogeneous structure in which different sub-regions have distinct functional roles (Moser and Moser 1998). There is a selective involvement of the HP in different types of fear memories and in different phases of fear learning, memory and extinction processes. For instance, hippocampal lesions before the acquisition session of fear conditioning selectively block contextual, but not cue fear memories (Phillips and LeDoux 1992). Similarly, hippocampal lesions one day after conditioning abolished in rats fear responses to a diffused contextual CS, but not to a discrete tone CS cue (Kim and Fanselow 1992; Anagnostaras, Maren et al. 1999). The HP seems to be crucial not only in acquisition and expression of fear memories, but also in extinction related processes. In fact, the reversible inactivation of the HP, by local infusion of muscimol, a γ-aminobutyric acid receptor type A ($GABA_A$) antagonist, impaired both acquisition and contextual encoding of fear extinction (Corcoran, Desmond et al. 2005), as well as contextual retrieval of fear memory after extinction (Corcoran and Maren 2001).

The HP revealed specific and sub-regional functions and a first distinction can be made between the dorsal part (dHP) which is more involved in spatial learning (Moser, Moser et al. 1993) and the ventral part (vHP), directly projecting to the amygdala and more related to emotional learning and memories, such as fear memories. In fact, lesion of vHP induces reduced fear expression (Kjelstrup, Tuvnes et al. 2002). Excitotoxic lesions of the vHP before and after training impaired acquisition and expression, respectively, of auditory trace fear conditioning, while similar lesions in the dHP had no effect on acquisition, but impaired expression (Yoon and Otto 2007). However other studies showed also involvement of the dHP in fear processes (Daumas, Halley et al. 2005). In the general scenario it appears that both the dHP and vHP are required for acquisition and maintenance of fear memories (Czerniawski, Ree et al. 2012), but in different aspects of conditioning, with the dorsal being involved

in temporal and contextual event representations and the ventral in aspects more strictly related to fear and anxiety (Esclassan, Coutureau et al. 2009).

A second level of analysis relates to the intra-hippocampal circuit, because it shows a defined and well-studied anatomy with specific functional properties in the fear process (Figure 2). The HP is a three sheet foiled structure that consists of the *cornu ammonis regions* (CA1, CA2 and CA3) and the *dentate gyrus* (DG); it receives a huge variety of inputs through the *entorhinal cortex* (EC) and sends its outputs from the CA1 via the subiculum. The flow of information, first described by Ramon y Cajal in the 1911 (Swanson and Swanson 1995), has at least two routes: one called the trisynaptic circuit in which the inputs arrive from the EC to the DG, then go to CA3 via the mossy fibre, finally reaching the CA1 via the Schaffer collateral connection. The other route is the monosynaptic direct connection between the EC and CA1 or CA3 directly (Witter, Groenewegen et al. 1989; Witter 1993). Specific inactivation or lesion studies in animals dissected the specificity of these sub-regions in the distinct fear memory processes and stimuli associations (reviewed in Langston, Stevenson et al. 2010). In contextual fear memories it has been shown that reversible inactivation of CA3 and CA1 has functional differences, being the CA3 necessary for elaboration and representation of the context and the CA1 involved in the consolidation process of contextual memory (Daumas, Halley et al. 2005). Another study, in which the function of these two areas has been assessed in the contextual specificity of a cue fear extinction process, shows that only lesions in the CA1, and not in CA3, made after extinction training, impaired the context dependence of extinction. Lesions in any of them before extinction training impaired the phenotype, with the conclusion that both CA1 and CA3 hippocampal areas are required for acquisition, but only CA1 is necessary for retrieval of context-dependent fear extinction (Ji and Maren 2008).

All together these studies advice a detailed and region-specific investigation of putative mechanisms at the hippocampal level that could be responsible for a pathological learned fear behavioural phenotype.

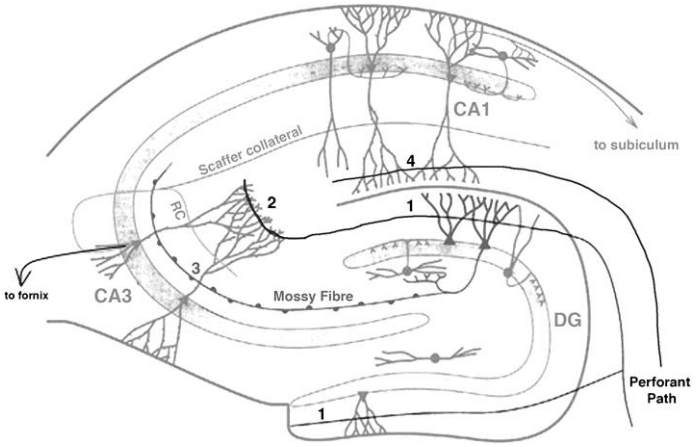

Figure 2: Hippocampal circuit. Schematic representation of the hippocampal circuit showing the trisynaptic route with input entering from the DG, and then to CA3 via the mossy fibre and then reaching the CA1 via the Schaffer collateral connection; the picture also shows the monosynaptic route with inputs from the EC to the CA1 via the perforant path. DG, *dentate gyrus*; CA3 and CA1, *cornu ammonis* 1 and 3; EC, *entorhinal cortex*. (Adapted from Daumas, Ceccom et al. 2009).

The Amygdala

The amygdala, “an almond-shaped” group of distinct nuclei within the temporal lobe (Kim and Jung 2006), is considered the core brain region of fear, including learned fear (Lavond, Kim et al. 1993; Davis 1997; Fendt and Fanselow 1999). It receives inputs from several brain areas (e.g. thalamus, cortex and hippocampus) and sends projections to a variety of autonomic and somatomotor structures (e.g. bed nucleus of stria terminalis, periaqueductal gray matter and hypothalamus), in order to gate fear responses. Therefore, it is of crucial importance to understand the intra-amygdala circuit to better investigate its function in fear learning (Figure 3). Within the intra-amygdala circuit distinct nuclei have been described. The basolateral part (BLA), often divided in basal (BA) and lateral (LA) portions, is thought to mediate the association established between the CS and the US in classical fear conditioning (Herry, Ciocchi et al. 2008) and the acquisition and expression of fear extinction (Sotres-Bayon, Bush et al. 2004; Herry, Trifilieff et al. 2006; Sotres-Bayon, Bush et al. 2007). The central amygdala (CeA) is divided into two sub-nuclei, the centro-lateral (CeL) and the centro-medial (CeM), and is essential for acquisition, expression and extinction of conditioned fear. A recent

optogenetic and pharmacological approach in mice has shown that fear responses are driven by output neurons in the CeM, which are under the local inhibitory control of CeL neurons (Ciocchi, Herry et al. 2010; Haubensak, Kunwar et al. 2010). Finally, other small but crucial nuclei in the amygdala are the lateral and medial intercalated cell clusters (lITC and mITC), which contain mostly interneurons that regulate the intra-amygdala circuit responsible for fear responses. These play a critical role in inhibiting fear when extinction occurs (Likhtik, Popa et al. 2008), through its extensive connections with the mPFC from which receive the "extinction" input (Royer and Pare 2002; Vertes 2004).

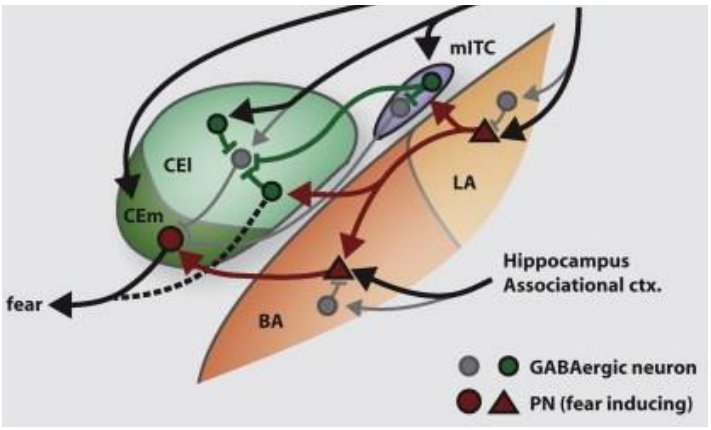

Figure 3: Amygdala circuit. Schematic representation showing the amygdala circuit and the principal neural players involved in gating fear responses. Briefly inputs enter to the BA, LA (although some direct route to the mITC and CeA as well occur), and from BA and LA are processed to the mITC and CeA. The mITC neurons inhibit CEl and CEm neurons and in the CeA circuit CEm neurons are the output neurons gating fear, under control of CEl interneurons. LA, lateral; BA, basal; mITC, medial intercalated cell cluster; CEl, centro lateral; CEm, centro medial; PN, pyramidal neurons. (Adapted from Ehrlich, Humeau et al. 2009).

The medial prefrontal cortex (mPFC)

The function of the mPFC has been extensively studied over the recent years. One of its main functions is "inhibitory" control over the amygdala to block fear (Sotres-Bayon, Bush et al. 2004; Sotres-Bayon and Quirk 2010). It contains different sub-regions, which play unique roles in fear learning and extinction, mainly the prelimbic (PL) and the infralimbic (IL) cortices that show opposite influences on fear expression in fear conditioning and extinction, respectively

(Gilmartin and McEchron 2005; Vidal-Gonzalez, Vidal-Gonzalez et al. 2006). In fact, IL muscimol inactivation prior extinction training did not affect fear memory recall but strongly impaired acquisition and expression of extinction, while similar inactivation of the PL reduced conditioned fear expression but had no effect on extinction (Sierra-Mercado, Padilla-Coreano et al. 2011). Regarding the specific role of the IL it has also been shown that pre-extinction training lesions selectively impaired extinction retrieval but not acquisition (Quirk, Russo et al. 2000). Of particular interest is the finding that just by pairing the CS with a brief IL stimulation in conditioned rats, fear response is reduced as if extinguished (Milad and Quirk 2002; Milad, Vidal-Gonzalez et al. 2004). All together these studies reveal a critical role of the mPFC, mainly from its ventral portion, the IL, in gating the inhibition of fear during the extinction process.

1.3 PAND definition and genetic factors

According to the Diagnostic and Statistical Manual of Mental Disorders, Fifth edition (DSM-V 2013), PAND is an anxiety disorder characterized by the experience of recurrent and unexpected panic attacks, which seem to occur “out of the blue”, without the existence of a clear threat. This concept was already postulated long time ago by Sigmund Freud: “anxiety attacks erupt suddenly into consciousness without being called further by any train of thought”, but recent studies showed that significant autonomic signs indeed precede the onset of attacks, although these are difficultly perceived by the patient (Meuret, Rosenfield et al. 2011).

The criteria for the diagnosis of PAND accounts not only with the experience of panic attack/s, but also consider specific cognitive symptoms related to the attacks themselves (table 2). A panic attack is defined in the DSM-V as a discrete period of intense fear or discomfort, in which four (or more) of a list of symptoms develop abruptly and reach a peak within ten minutes (table 3).

Table 2: Key criteria for a clinical **diagnosis of PAND** (adapted from DSM-V 2013).

Diagnostic criteria for PAND
Recurrent unexpected panic attacks
At least one of the attacks has been followed by 1 month of one of the following: a. Persistent concern about having additional attacks b. Worry about the implication of the attack or its consequences c. Significant change in behavior related to the attack
Presence or absence of agoraphobia (PAND with or without agoraphobia)
The panic attack are not due to the direct physiological effects of a substance (e.g. drug of abuse, medication) or a general medical condition (e.g. hyperthyroidism)
The panic attacks are not better accounted for by another mental disorder, such as social phobia (e.g. occurring on exposure to feared social situation), specific phobias (e.g. on exposure to a specific phobic situation), obsessive-compulsive disorder (e.g. on exposure to dirt in someone with an obsession about contamination), posttraumatic stress disorder (e.g. in response to stimuli associated with a severe stressor), or separation anxiety disorder (e.g. in response to being away from home or close relatives).

Table 3. Panic attack associated symptoms (adapted from DSM-V 2013).

Symptoms developed during a panic attack	
Palpitation and/or accelerated heart rate	De-realization (feelings of unreality) or depersonalization (being detached from oneself)
Dry heaving and/or gagging	Fear of losing control or going insane
Sweating	Sense of impending death
Trembling or shaking	Paresthesias (numbness or tingling sensations)
Sensations of shortness of breath or being smothered	Chills or hot flashes
Feeling of choking	Tinnitus
Chest pain or discomfort	Neck soreness
Nausea or abdominal distress	Headache
Feeling dizzy, unsteady, lightheaded, or faint	Uncontrollable screaming or crying

The development of PAND is a complex pathophysiological process that finds already several aetiological factors contributing since early developmental stages. In fact PAND is a complex disorder in which many different genetic factors may contribute to a genetic predisposition (Kendler 2005). Human genetic studies have reported more than 20 different genes conferring susceptibility or modulating the pathology of PAND. However most of these studies fail to find replication in larger cohort sets or in different populations, probably because of its polygenic nature, with different genes providing a small contribution to the phenotype either by interaction or by having an additive effect. The identified genes belong to different biological pathways and enclose systems such as the monoaminergic, neuropeptides or neurotrophins (reviewed by our group in Santos, D'Amico et al. in preparation). Genes of the serotonergic system such as the serotonin transporter (*5-HTT*) (Maron, Lang et al. 2005; Strug, Suresh et al. 2010), the serotonin receptor type-2A (*HTR2A*) (Inada, Yoneda et al. 2003) and the tryptophan hydroxylase 2 (T*PH2*) (Kim, Lee et al. 2009) were reported as susceptibility factors predisposing to PAND, suggesting a role of the serotoninergic system in PAND.

Neuropeptide Y (*NPY*) was also reported in human PAND studies (Domschke, Hohoff et al. 2008) and another interesting candidate gene is the gastrin-releasing peptide (*GRP*), associated with PAND in human genetic studies (Hodges, Weissman et al. 2009). Neuropeptides and related proteins, like the GRP, regulate the sympathetic nervous system and appears to modulate stress, fear and anxiety responses (reviewed in Moody and Merali 2004).

Several studies reported the *NTRK3* gene, encoding for the high affinity receptor for NT3, TrkC, as a candidate gene for PAND. A genetic duplication on chromosome 15 (DUP 25), where *NTRK3* is located, has been found in PAND patients (Gratacos, Nadal et al. 2001), although this association has not been replicated by other studies (Schumacher, Otte et al. 2003; Henrichsen, Delorme et al. 2004; Zhu, Bartsch et al. 2004). These contradictory findings are probably due to the heterogeneity of genetic factors contributing to PAND and population background. In agreement with *NTRK3* potential role in PAND, subsequent studies have shown other alterations, such as single nucleotide polymorphism

in the regulatory 5'UTR region of the *NTRK3* gene, inducing alterations in the TrkC mRNA expression levels (Armengol, Gratacos et al. 2002), as well as allele variants in microRNA target sites of the *NTRK3* gene (Muinos-Gimeno, Guidi et al. 2009). All together these studies suggest a direct involvement of the neurotrophic system, especially to deregulation of the *NTRK3* gene to the pathophysiology of PAND.

1.4 PAND fear memories

The post-attack complications and attack-related cognitive processes probably underlie the development of altered and exaggerated fear memories and this represents a major problem poorly investigated in PAND. One possibility is that the panic attack causes the conditioning to exteroceptive and interoceptive cues to which it is associated, a classical associative fear memory symptom (Bouton, Mineka et al. 2001) and experimentally enhanced fear conditioning has been shown to be a recurrent feature in anxiety disorder, including PAND (for review see (Lissek, Powers et al. 2005). PAND cognitive phenotypes range from the enhanced response to conditioned stimuli associated to aversive experiences, (Wolpe and Rowan 1988) to more complex and demanding affected cognitive profiles. PAND patients showed impaired ability to discriminate safe vs. danger cues (Lissek, Rabin et al. 2009), which has been postulated to often generate a generalization of fear memories (Lissek, Rabin et al. 2010). Being the unpredictability of a panic attack a key factor in the induction of stronger fear memories in PAND, it has been proposed that this could be due to a deficit in declarative associative learning when a signaling CS is separated to a non-aversive US by a trace time interval, as in the classical trace eyeblink conditioning (Grillon, Lissek et al. 2007). Interestingly, in a visual contextual fear conditioning paradigm PAND patients showed no differential response as compared to healthy controls during the acquisition of a CS+ (neutral picture) / US (electrical stimulus) association, while they did show a stronger peripheral signs of fear (skin conductance) and a more negative evaluation (valence rating) of the CS+ during extinction, revealing an enhanced resistance to fear extinction (Michael, Blechert et al. 2007).

Being PAND a fear disorder in which the cognitive-related processes seem to represent the central pathopsychological signs to be understood and tackled, it is crucial to study how fear memories are generated, consolidated and extinguished. To this aim a detailed "flowchart" of the different cognitive domains has been recently proposed as a guideline to investigate specific phases of fear memory and thus might help to understand which domain is primarily or mainly affected in fear disorders. (Figure 4 adapted from Parsons and Ressler 2013).

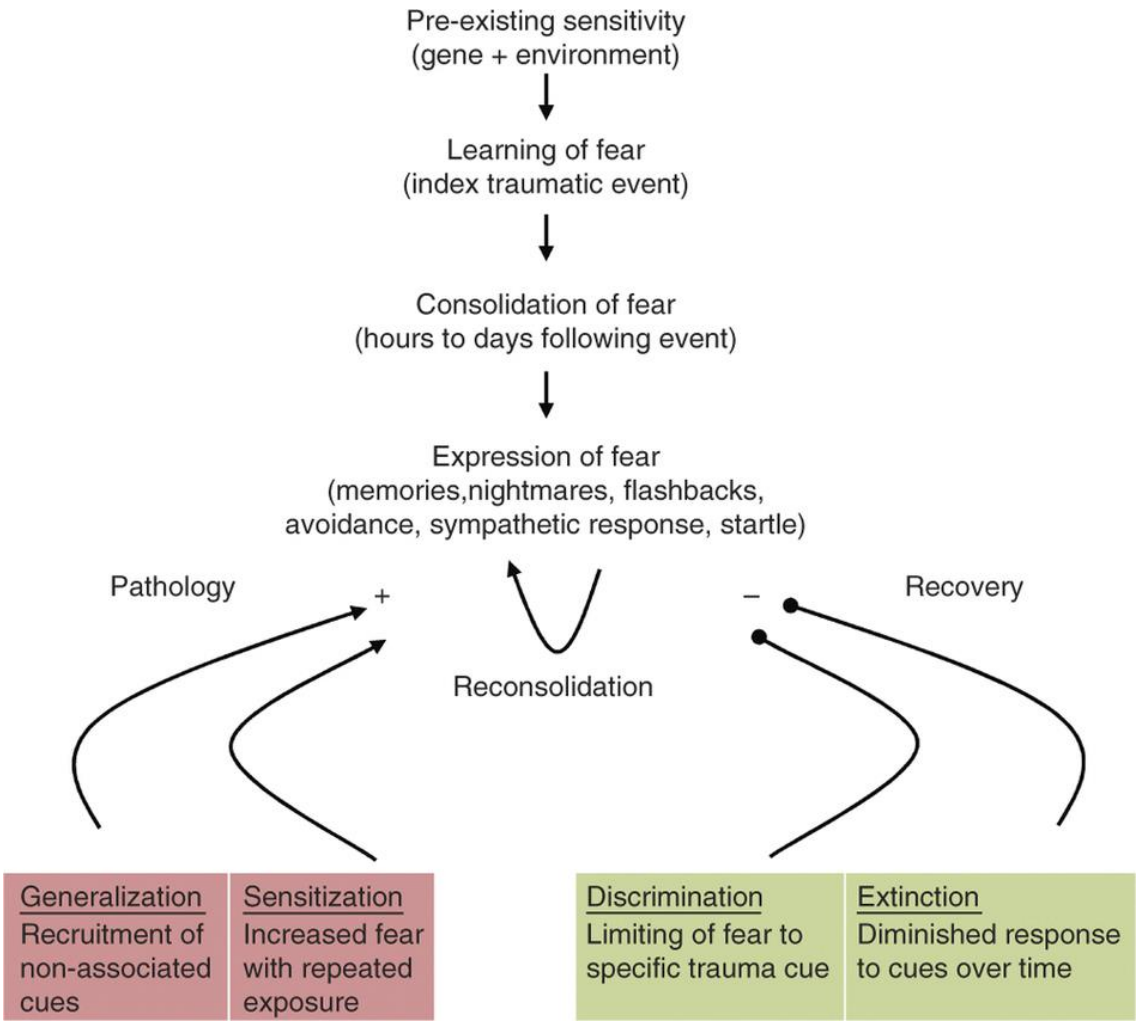

Figure 4: Model for development, establishment and intervention in a fear disorder. Genetically predisposed individual, in certain environmental conditions, experience a traumatic event and thus learn to fear to those cues associated with the trauma. During a subsequent period the memory of the trauma and the associated cues are consolidated and might re-appear in several different forms. This recurrent fear memory expression might be sensitized in those individuals developing a fear-related disorder and, in some case, may be generalized to other

cues not associated to the trauma before. In normal individuals, fear memory of a trauma extinguishes over time and discrimination occurs to selectively fear to those cues associated to the traumatic event and not to others. In people developing a fear disorder extinction and discrimination might be impaired and potentially targeted for therapy. (Adapted from Parsons and Ressler 2013).

1.5 The disrupted fear circuit in PAND patients

By comparing PAND patients with healthy controls several studies have tried to dissect the brain areas involved in this pathology. An old literature revision by Gorman and colleagues (Gorman, Kent et al. 2000) proposed a neuroanatomical hypothesis of PAND, which implicates several brain regions in hallmark aspects of the disorder. Among them are loci in the brainstem and the hypothalamus, which controls panic attacks and stress response, the limbic system given its involvement in anxiety and exaggerated fear and cortical areas, which are more related to phobic reactions and emotional regulation.

A recent revision of the last decade neuroimaging studies in PAND patients by our group (Santos, D'Amico et al. in preparation) highlights the involvement of precortical and limbic brain areas. Structural magnetic resonance imaging (MRI) studies have revealed controversial results regarding the hippocampus alterations in patients, with some studies reporting volume reductions, others increases and others no differences in comparison to healthy controls (Vythilingam, Anderson et al. 2000; Massana, Serra-Grabulosa et al. 2003; Uchida, Del-Ben et al. 2003). Metabolic studies have reported abnormalities in benzodiazepine receptor, a GABA agonist often used in patients (Bremner, Innis et al. 2000; Hasler, Nugent et al. 2008).

In the amygdala MRI studies reported reduced volume in PAND patients (Uchida, Del-Ben et al. 2003; Hayano, Nakamura et al. 2009) and functional MRI (fMRI) studies described reduced amygdala activation in PAND patients in front of fearful faces (Pillay, Gruber et al. 2006) and no alterations in front happy faces (Pillay, Rogowska et al. 2007). In general the amygdala alteration in patients with PAND could represent a central neuroanatomical and functional marker of the disorder and need to be always taken into account in future research studies (Kim, Dager et al. 2012).

Concerning the prefrontal cortical areas, several regions have been found to be altered in PAND patients, such as the anterior cingulate cortex (ACC), the orbitofrontal cortex (OFC) and the insula, which are considered as central brain checkpoints for somatic and cognitive symptoms in anxiety disorders (Malizia 1999). The ACC is reduced in volume (Asami, Hayano et al. 2008) and aberrantly activated in response to safe vs. threatening stimuli, possibly reflecting an incapacity in the evaluation of stimuli valences (Chechko, Wehrle et al. 2009); the OFC has been found generally reduced in volume (Lai and Wu 2012) and hypo-functioning (Kent, Coplan et al. 2005) and the insula, which drive bodily sensations and interpretations to regulate emotional responses (Critchley, Mathias et al. 2001) has been found altered in its gray matter volume in PAND patients, but with opposite findings depending on the study (Uchida, Del-Ben et al. 2008; Asami, Yamasue et al. 2009; Lai and Wu 2012).

To sum up, all the neuroimaging studies in PAND revealed, sometimes with controversial results, significant alterations in the hippocampus-amygdala-prefrontal cortical brain fear circuit. The technical limitations of these approaches do not allow for defined understanding of the possible alterations in term of sub-regions and cell type specificity. This could be more easily achieved with the use of validated animal models, an efficient tool for translational pathophysiological discovery.

1.6 Players controlling fear memories: focus on glutamatergic and GABAergic systems

A critical point to take into account in the search for the neural mechanisms involved in pathological fear memories is the contribution of specific neurotransmitters and cell types within the fear circuit and their putative alterations underlying disturbed fear. To this point we here describe the contribution of the excitatory (glutamatergic) and inhibitory (GABAergic) systems to fear memories, which together and in a co-working way allow for dynamic and activity-dependent plastic functions that are required for learning and memory processes.

It has been recently shown that electrophysiological stimulation of the hippocampus CA1 region in mice induced responses in both excitatory and inhibitory synapses, maintaining a homeostatic balance of excitatory and inhibitory inputs (Bourne and Harris 2011). A similar balanced excitatory-inhibitory mechanism has also been described in the DG-CA3 intra-hippocampal circuit upon stimulation of the mossy fiber tract (Trevino, Vivar et al. 2011). The natural explanation of a balanced functioning of these two systems in the hippocampus can be found in the hystological distribution of glutamatergic and GABAergic neurons, extremely interconnected in a tight network. DG glutamatergic neurons send their projections to the CA3 region. In CA3, glutamatergic neurons are localized in the *pyramidal layer* (Py) and their dendrites arborize and make synaptic contacts at the level of the *stratum radiatum* (Rad) both with DG projecting axons and with axons coming from local CA3 inhibitory interneurons (Neves, Cooke et al. 2008). These GABAergic interneurons are mostly localized in the same Rad and in the upper *stratum oriens* (Or) *layer* and, besides their inhibition of pyramidal neurons, they are also mutually controlled by other local inhibitory interneurons (Chamberland and Topolnik 2012). A very similar cellular distribution is found in the CA1 region of the HP in which the same three layers can be identified, the Py, containing mainly excitatory neurons and the Or and Rad containing interneurons (Oliva, Jiang et al. 2000). In CA1 the input information arrives from CA3 projecting pyramidal neurons establishing axonal-dendrite synaptic contacts with CA1 pyramidal neurons in the Rad. Here CA1 pyramidal neurons also receive inhibitory contacts from local interneurons (Klausberger 2009). The dendtric arborization of CA1 pyramidal neurons and their contacts with different excitatory and inhibitory cell types, contributes actively to the input/output integration, transformation (Hausser, Spruston et al. 2000; Harvey and Svoboda 2007) and translation to downstream brain regions, such as the mPFC and the amygdala. To these targets some CA1 pyramidal neurons send simultaneously axonal projections (Ishikawa and Nakamura 2006), suggesting their central role in the fear circuit.

In the amygdala the excitatory and inhibitory neurons are distributed in an organized way. Their inter-neuronal interactions allow for the functional flow of information and are specifically activated during fear processes. In acquisition of fear memory, inputs arriving from the BLA can induce activation of a population of glutamatergic neurons, representing here the majority, although also local GABAergic interneurons can be found (McDonald 1982). Consequently BLA glutamatergic neurons activate fear output in the CeM part. In parallel processes, fear acquisition and expression can be switched on through direct inputs to the CeA, where the majority of neurons are GABAergic (Cassell, Gray et al. 1986; Swanson and Petrovich 1998), by either increasing activation of CeM neurons or by control of CeL neurons to reduce their inhibition to the CeM.
During extinction inputs can lead to increased activity of fear-inhibiting projection neurons in the BLA (interneurons) and/or direct activation of the mITC, a specialized cluster of GABAergic interneurons that gate interactions between the BLA and the CeA (Millhouse 1986); both these pathways result in a decreased activation of fear output neurons in the CeM to suppress fear (Ehrlich, Humeau et al. 2009). To the fine mechanism involved in the amygdala excitatory-inhibitory circuit, it has been nicely proposed a mathematical model in which a balance between pyramidal cells and interneurons activation in the LA is controlling both acquisition and extinction of conditioned fear (Li, Nair et al. 2009).
Finally, in the mPFC the two glutamatergic and GABAergic systems also play an important “game” in control and direct the inhibition of fear when needed. The way of communication between the mPFC and the amygdala, required for extinction of fear responses, is through multiple neural long range projections; in fact IL excitatory neurons send strong input directly to the LA and ITC nuclei of the amygdala, while PL excitatory neurons do so to the BA part, and both directly contact the CeA (Berendse, Galis-de Graaf et al. 1992; McDonald, Mascagni et al. 1996). These mPFC-Amy connections suggest the direct influece of the mPFC onto amygdala circuit via glutamatergic transmission. However locally in the mPFC are found multiple type of GABAergic neurons and these receive projections from the amygdala, so operating a modulatory

function in the amygdala-mPFC activity (Cunningham, Bhattacharyya et al. 2008).

To a more broad level, genetic manipulations that either enhanced the glutamatergic or depleted the GABAergic systems have reported enhancement of fear memories. A detailed revision of these studies has recently been done by our group (Santos, D'Amico et al. in preparation) and here we describe the most significant examples.

Mice lacking alpha 4 subunit $GABA_A$ receptor, which results in less inhibition to the network, show enhanced trace and contextual fear memories (Moore, Cushman et al. 2010). Moreover, the ablation of glutamic acid decarboxylase 65 gene (*Gad65*, the rate-limiting enzyme in the synthesis of GABA), results in reduced fear responses such as freezing but increased flight and escape responses, in fear conditioning paradigms (Stork, Yamanaka et al. 2003). Interestingly, these findings have a parallel in PAND patients, suggesting in part that alterations of the GABA function may play an important role in the pathophysiology of PAND. Specifically, decreased GABA levels were found in ACC and basal ganglia of PAND patients (Ham, Sung et al. 2007) and pharmacological agents that activate the GABAergic system are, indeed, effective to reduce pathological fear in human PAND patients, like benzodiazepines, $GABA_A$ receptor agonists (Susman and Klee 2005) or tiagabine, a GABA reuptake inhibitor (Zwanzger and Rupprecht 2005).

The contribution of the glutamatergic system to fear responses has been reported (LeDoux 1994; Walker, Ressler et al. 2002). Mice overexpressing the N-methyl-D-aspartate (NMDA) glutamate receptor subunit 2B gene (*Nr2b*) show enhanced contextual and cue fear memory and faster extinction (Tang, Shimizu et al. 1999). The impaired glutamate transmission has also been hypothesized in anxiety disorders, including PAND (Harvey and Shahid 2012) and several pharmacological approaches targeting the glutamatergic system are being successfully employed in the treatment of fear-related disorders, using antagonists of glutamate receptors such as NMDA, A-Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic Acid (AMPA) and metabotropic mGluR (for extensive review see Cortese and Phan 2005; Harvey and Shahid 2012; Riaza

Bermudo-Soriano, Perez-Rodriguez et al. 2012), therefore supporting the role of the glutamatergic system in fear.

1.7 Neurotrophins and their involvement in fear memories and PAND

Neurotrophins and their receptors are a family of protein regulating many processes in both developing and adult central nervous system (CNS), such as cell proliferation, apoptosis, differentiation, as well as neural dendritic and axonal growth and plasticity. Several neurotrophins have been identified including the nerve growth factor (NGF), the brain-derived neurotrophic factor (BDNF), neurotrophin 4/5 (NT 4/5) and NT3 (Bibel and Barde 2000; Chao 2003; Lu, Pang et al. 2005). Each neurotrophin binds with high affinity to specific transmembrane receptors that belong to the family of neurotrophin tyrosine kinase receptors (Trk): TrkA is the receptor for NGF, TrkB for BDNF and NT4/5 and TrkC for NT3 (Patapoutian and Reichardt 2001; Huang and Reichardt 2003; Reichardt 2006). Besides, neurotrophins can also bind with low affinity to the shared p75 neurotrophin receptor (p75 NTR) and cross-bind to other members of the Trk family, as the case of NT3 which show cross-reactivity with both TrkA and TrkB. (Lee, Kermani et al. 2001). And finally also interaction between Trk and p75 receptors can lead to changes in the binding affinity to neurotrophins (Esposito, Patel et al. 2001) (Figure 5).

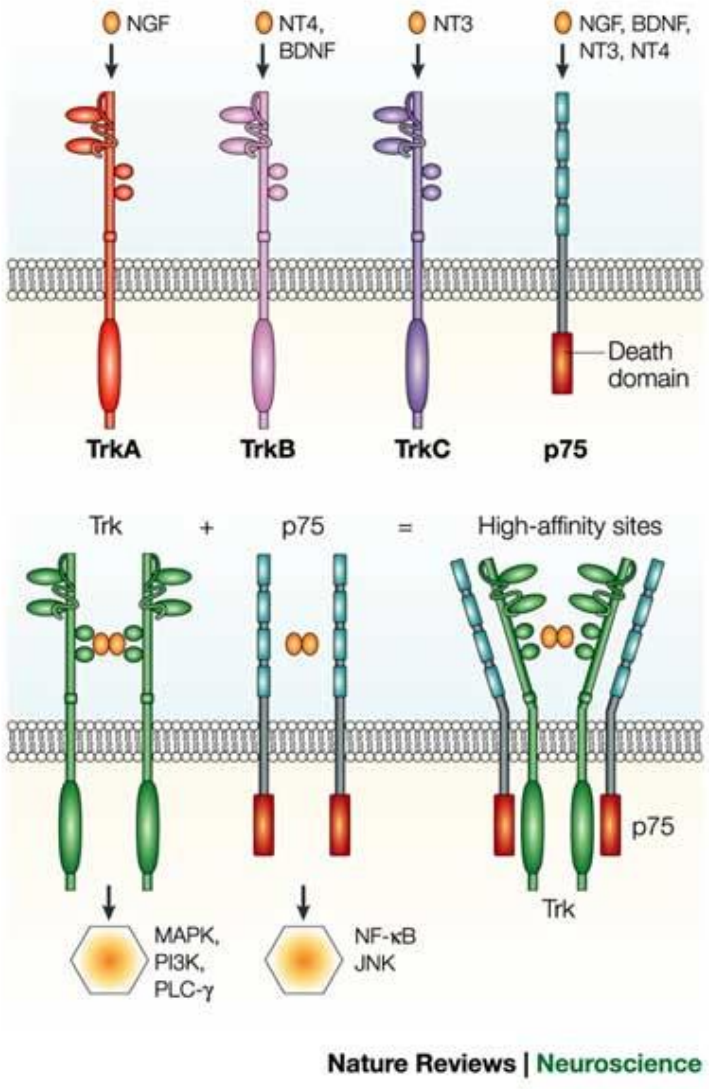

Figure 5: Neurotrophins and their receptors. Neurotrophins bind with high affinity to Trk receptors and with low affinity to the p75 NTR. Specific intracellular pathways are activated upon specific binding and conditions. NGF, nerve growth factor; NT4, neurotrophin 4; BDNF, brain derived neurotrophic factor; NT3, neurotrophins 3; TrkA, B and C, tyrosine kinase receptor A, B and C; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositide 3 kinase; PLC-γ, phosphoinositide phospholipace C type γ; NF-$_k$B, nuclear factor k activated B cells; JNK, c-Jun N-terminal kinases. (Adapted from Chao 2003).

This differential binding to the Trk or p75 receptors implicates different functions, via activation of specific intracellular signaling pathways (Figure 6). In fact the major pathways activated upon Trk receptor activation are the mitogen-activated protein kinase (MAPK) phosphorylation cascade, which promote

neuronal differentiation and plasticity, the phosphatidylinositide 3 kinase-protein kinase B (PI3K-Akt), involved in neuronal growth and survival and the inositide 3-phosphate-protein kinase C (IP3-PKC), which lead to intracellular Ca^{2+} mobilization. For the p75 NTR other pathways are engaged such as the c-Jun N-terminal kinases-cJUN (JNK-cJUN) leading to apoptosis or the Nf-$_k$B, promoting survival (Chao 2003; Reichardt 2006).

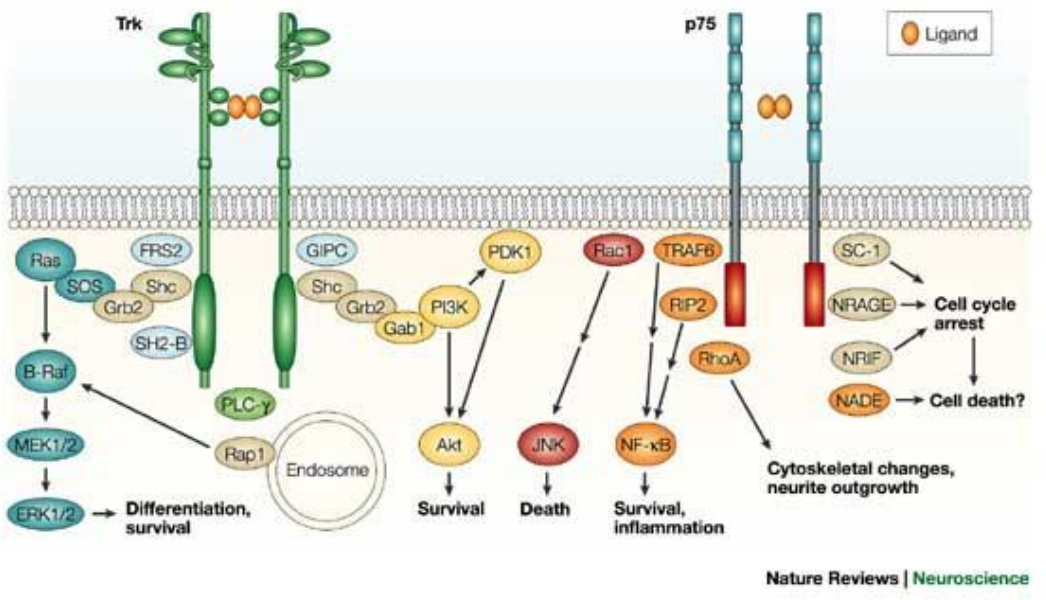

Figure 6: Trk and p75 NTR mediated intracellular signaling pathways. Several different signaling pathways can be engaged upon binding of neurotrophins to Trk or p75 receptors, leading to different functions such as differentiation, survival, death, or neurite modifications. (Adapted from Chao 2003).

Gene expression, protein synthesis, post-translational modifications, intracellular packaging, transport and secretion are all key regulatory mechanisms that allow neutrophins and their receptors to fine tunning many neural processes including plasticity, a mechanism underlying learning and memory. Thus alterations of the neurotrophin system may account for CNS dysfunction. In fact, members of these families have been implicated in anxiety disorders and altered fear behaviours. Chen and colleagues (2006) have shown that mice carrying a common single nucleotide polymorphism in the *BDNF* gene

(Val66Met) show increased anxiety-like behaviour and impaired fear extinction learning (Frielingsdorf, Bath et al. 2010; Soliman, Glatt et al. 2010). The same variant has been associated to the posttraumatic stress disorder, an anxiety disorder sharing common fear phenotype with PAND (Zhang, Benedek et al. 2013). Additionally, *Bdnf* heterozygous mice show impaired contextual fear memory, but intact cue fear learning (Liu, Lyons et al. 2004). In another study, *in vivo* infusion of BDNF within the rat HP-IL fear circuit is sufficient to induce extinction of fear memory, by acting on the glutamatergic system (Peters, Dieppa-Perea et al. 2010).

The *NTRK3* gene has been strongly proposed as a genetic factor contributing to PAND (see introduction section 1.3). The genetic alterations described in PAND patients would, high probably, direct a susceptibility to the enstablishment of the PAND, by disrupting the highly regulated system of the NT3-TrkC pathways. In fact it is widely known that TrkC receptor, upon NT3 binding, dimerizes and transphosphorylates tyrosine residues in their own cytoplasmatic kinase domain, activating multiple downstream signaling cascades (Huang and Reichardt 2003). One of these cascades is the MAPK, finally converging to activation of the extracellular signal-regulated kinase (ERK) (English, Pearson et al. 1999; Pearson, Robinson et al. 2001; Reichardt 2006).

ERK signalling has been shown to be involved in hippocampus-dependent learning and memory processes (Peng, Zhang et al. 2010), particularly fear conditioning and fear extinction (Atkins, Selcher et al. 1998; Fischer, Radulovic et al. 2007; Tronson, Schrick et al. 2009). Moreover, the NT3-TrkC pathway seems to actively modulate the glutamatergic system by favouring the establishment of excitatory synaptic contacts. It increases excitatory current through their modulation of presynaptic neurotrasmitter containing vesicles (Collin, Vicario-Abejon et al. 2001). Exogenous application of NT3 (and BDNF) to hippocampal and neocortical neurons can enhance glutamatergic synaptic transmission via activation of TrkC (and TrkB) receptor, by increasing the efficacy of glutamate release (Lessmann 1998). Also, a recent study reported a direct function of the TrkC receptor at the postsynaptic site as a mediator for the

specification and organization of excitatory synaptic complexes (Takahashi, Arstikaitis et al. 2011).

In conclusion neurotrophins and especially TrkC, are involved in the pahopysiological features of anxiety disorders, such as PAND and are actively participating in the modulation of the excitatory system, among their several functions. Thus, they seem to play a role in fear learning and memory processes. All that makes the *NTRK3* gene one of the best candidate gene for the understanding of PAND fear altered phenotype and the putative mechanisms underlying it.

1.8 The Tg*NTRK3* mouse model of PAND

The transgenic Tg*NTRK3* mouse has been generated in our laboratory in the past and it has been validated as a model for PAND (Dierssen, Gratacos et al. 2006). Briefly, Tg*NTRK3* mice overexpress the human *NTRK3* gene under the control of the platelet-derived growth factor subunit beta (PDGFβ) promoter driving the expression into neurons (Dierssen, Gratacos et al. 2006). Detailed TrkC expression analysis of Tg*NTRK3* brains revealed no ectopic expression, based on normal endogenous expression patterns (Lamballe, Smeyne et al. 1994), but increased total mRNA and protein levels from total brain extracts (Dierssen, Gratacos et al. 2006). However, probably due to tight expression regulation Tg*NTRK3* mice showed a complex picture of expression in specific brain areas (Dierssen, Gratacos et al. 2006).

Hallmark features of PAND are reflected in the Tg*NTRK3* mouse model. They showed a) face validity, with increased anxiety-like behaviour and panic-like reactions; b) predictive validity, as their anxiety phenotype was reversed in a dose-dependent manner with treatment with diazepam; c) construct validity, with alteration in the noradrenergic (NA) system (Dierssen, Gratacos et al. 2006) and altered spontaneous firing rate of naradrenergic neurons in the locus coeruleus (LC), a downstream brain area activated by the amygdala (Gallego, Murtra et al. 2010).

Furthermore, studies revealed other phenotypic and pharmacological significative features in Tg*NTRK3* mice such as their specific and selective responsiveness to panicogenic drugs, such as sodium lactate, a widely used drug to induce panic attacks in humans (Johnson, Truitt et al. 2008) and animals (Bergold, Pinkhasova et al. 2009). Sodium lactate in Tg*NTRK3* mice enhanced their panic-like phenotype in the mouse defense test battery (MDTB). Moreover, they are differentialy activated, as compared to wild type mice, in fear related brain regions upon treatment with other panicogenic drugs, such as caffeine or yohimbine (Sahun, Gallego et al. 2007). And finally Tg*NTRK3* mice showed a enhanced anxiety behaviour under chronic environmental stress (high illuminated housing condition) (Amador-Arjona, Delgado-Morales et al. 2010), which has been longly considered a major risk factor to establish neuropsychiatric disorders, including PAND (Schreiber, Lauer et al. 1996).

At the level of the hippocampus, a key region in the fear circuit, previous findings have shown some important dysfunctions, such as increased neural density in all hippocampal subregions and reduced glia density mostly in the CA1 and the CA2 subregions, along with increased levels of the NMDA receptor subunits NR1 and NR2B in their unphosphorilated (not activated) isoforms (Sahun, Delgado-Garcia et al. 2007).

Interstingly it has to be considered that in the CA1, CA2 and CA3 hippocampal fields, possibly through negative feedback mechanisms, Tg*NTRK3* mice showed also reduction in the expression levels of the TrkC protein (Dierssen, Gratacos et al. 2006). But more importantly to understand the potential hippocampal-dependent alterations in fear memory is the finding that Tg*NTRK3* mice showed a huge enhancement and persistent in time of the CA3-CA1 long term potentiation (LTP), evoked by high frequency stimulation (HFS) and measured in conscious mice. Moreover, the Tg*NTRK3* mice enhanced CA3-CA1 excitatory postsynaptic potential (fEPSP) was completely dissociated to their impaired acquisition and extinction of a classical CS-trace-US eyeblink conditioned response (Sahun, Delgado-Garcia et al. 2007).

All these data suggest a critical role of TrkC in hippocampal-dependent functions and in the manifestations of signs typical of PAND, which make the

Tg*NTRK3* mouse an usefull model to further investigate fear memory disturbances.

Being PAND a fear-related disorder and Tg*NTRK3* a validated mouse model of PAND, we based our study on the hypothesis that a key pathophysiological domain in human PAND patients is the development of aberrant learned fear memories. These may rely on an abnormal functioning of the brain fear circuit implicated in this process. Thus, the study of this circuit in the Tg*NTRK3* mice during fear memories might contribute to define potential mechanisms and novel targets relevant to PAND fears.

HYPOTHESIS AND OBJECTIVES

2. HYPOTHESIS AND OBJECTIVES

2.1 Hypothesis

The proposed genetic predisposition to develop PAND confered by the *NTRK3* gene suggest a potential involvement of the neurotrophin system and its functionality in pathological fear. Our working hypothesis proposes that the genetic deregulation of the TrkC neurotrophic receptor would affect the fear-related cognitive processes in PAND patients, disrupting modulation and dynamic adaptive changes of the fear brain circuit.

2.2 Objectives

To address our hypothesis we used the genetically modified transgenic mouse model of PAND, the Tg*NTRK3*, overexpressing the human *NTRK3* gene. The general objective of this doctoral thesis was to characterize fear learning and memoy processes and the potential molecular and cellular mediators in the brain fear circuit, aiming at finding novel mechanisms and thus potential targets for future therapy.

To achive this general aim the following concrete objectives were proposed:

- To characterize fear memory responses and to highlight specific phases in fear learning.
- To map the activation pattern of the fear brain circuit during fear memory processes.
- To identify potential molecular and cellular mechanisms in the hippocampus, as a brain region involved in fear learning and cognition.
- To understand the involvement of the neurotrophin system, supporting perturbed fear memory.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 Animals

In all the experiments, we used young adult (two to four months old) WT and Tg*NTRK3* male animals. Tg*NTRK3* mice, generated several years ago in our laboratory, overexpress the human *NTRK3* gene under the control of the neuron-specific platelet-derived growth factor (*PDGF*) promoter, and were validated as a model of PAND (Dierssen, Gratacos et al. 2006). The colony is maintained by crossing transgenic Tg*NTRK3* males with C57Bl/6SJL females, purchased from Jackson laboratory (ME, USA).

In the context of this Thesis, we generated a double transgenic line, Thy1-YFP/*NTRK3*, by crossing Tg*NTRK3* animals with B6.Cg-Tg(Thy1-YFP)HJrs/J transgenic mice (stock number: 003782, the Jackson laboratory), expressing the reporter gene YFP under the *Thy1* promoter.

The WT littermates served as controls for all the experiments. Pups were weaned at postnatal day 21 and group housed (3-5 animals) by sex in standard laboratory cages filled with sawdust. Mice were maintained in the animal facility of the PRBB (Barcelona Biomedical Research Park, www.prbb.org) with controlled temperature at 22 °C, on a 12h light/ 12h dark cycle with standard food pellets (Mucedola, MI, Italy) and water *ad libitum*.

All the experiments were performed in accordance with the European Communities Council Directive, 86/609/EEC and all the procedures were approved by the local ethical committee (CEEA: JMC-07-1001-MDS; MDS-08 1116; MDS-09-1165; MDS-09-1165; MDS-13-1492). All the experimenters were qualified to work with laboratory animals.

3.2 DNA extraction and genotyping

DNA was extracted from tail tips using the saline method. Briefly, NaOH 50 mM was added to the tail tips and incubated at 98 ºC for 30 minutes, followed by vortex mix with Tris-HCL 1M pH 8.0. Finally, DNA-containing supernatant was obtained after centrifugation (6 minutes at 13.000 rpm). Genotypes were

determined by polymerase chain reaction (PCR) according to the protocol established in the laboratory for the Tg*NTRK3* line (Dierssen, Gratacos et al. 2006) using the primer pairs:

Forward*: NTRK3* hum/mou-F 5′-CTGTTTGACGAAGTGAGTCCC-3′

Reverse*: NTRK3* hum/mou-R 5′-TCCAGTGACGAGGGCGTG-3′

Genotyping of the Thy1-YFPH/*NTRK3* line was performed for the presence of the human *NTRK3* transgene and for the presence of YFP transgene, as protocol provided by Jackson laboratory and using the following primers:

Transgene reverse: 5'-CGGTGGTGCAGATGAACTT-3'

Transgene forward: 5'-ACAGACACACACCCAGGACA-3'

Internal control reverse: 5'-GTAGGTGGAAATTCTAGCATCATCC-3'

Internal control forward: 5'-CTAGGCCACAGAATTGAAAGATCT-3'

3.3 Behavioural experiments

Fear learning procedures

Behavioural responses during learning processes are the manifestations of dynamic biological processes. It represents a great challenge to behavioural neuroscientists to understand what information is acquired in a particular learning session and/or assay. One way of elucidating mechanisms involved in discrete learning sessions is to study associative learning, defined as an adaptive process that allows an organism to learn to anticipate events.

One classical form of associative learning that has gained popularity is fear conditioning. The typical animal behavioural response is freezing, that takes place following pairing of an US, such as foot shock, with a CS, a particular context and/or a cue (Figure 1).

Two types of conditioning that are typically employed are contextual and cue (delay or trace) fear conditioning. Contextual conditioning is induced by administering the US in a specific context, while in cue conditioning the US is anticipated by a salient stimulus, such as a tone. In delay conditioning the US is administered to co-terminate with or occur immediately after the CS and in trace conditioning an empty interval (trace) separates the CS from the onset of the

US. Trace conditioning adds additional complexity to delay conditioning, as the time interval between the CS and US requires the formation of a temporal relationship between the two stimuli.

Testing animal behaviours in these different paradigms allow for the analysis of specific cognitive domains related to associative learning and memory.

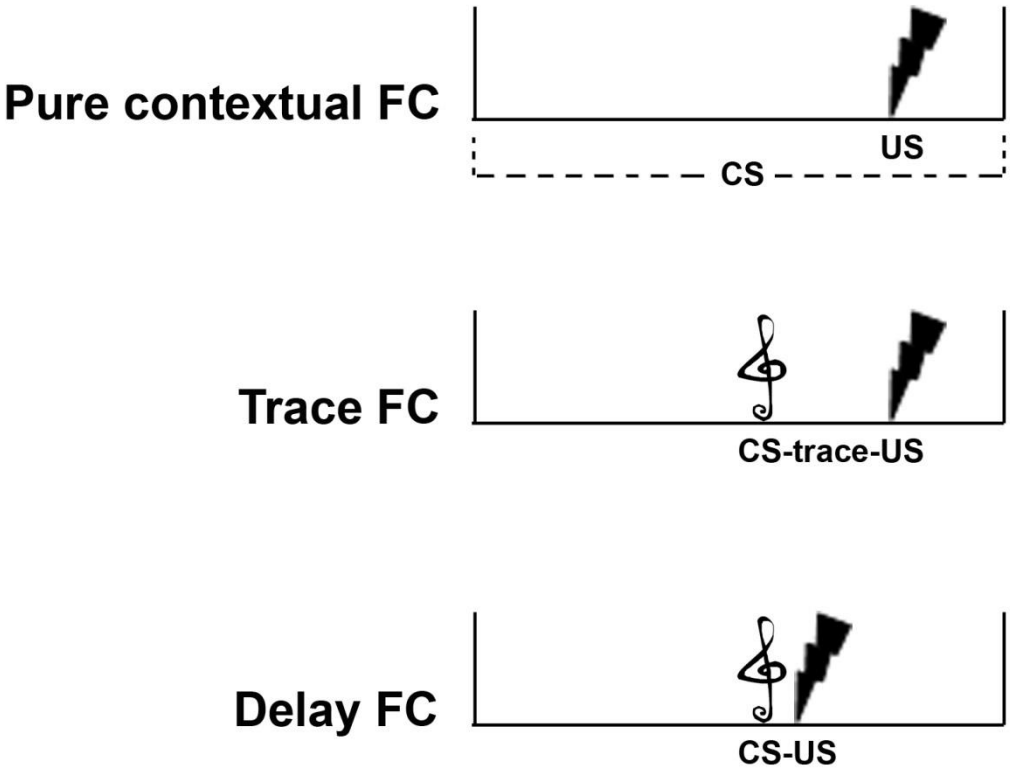

Figure 1: Fear Conditioning paradigms. Schematic representations of the different FC behavioural paradigm used. Pre contextual FC, in which a neutral context, serving as CS is paired with the aversive foot shock (US); In the trace FC the CS is a tone and is associated with the US, with a trace time interval in between. Lastly the delay FC in which a tone (CS) is immediately followed by the US. FC, fear conditioning; CS, conditioned stimulus; US, unconditioned stimulus.

3.3.1 Fear conditioning paradigm

Fear conditioning paradigms were performed in an apparatus composed of a metal cage (20 cm x 20 cm x 25 cm) with a grid floor connected to a shock generator, inside a sound-attenuating box (StartFear, Panlab Harvard Apparatus, Barcelona, Spain). Freezing behaviour, defined as lack of

movement except for respiration for at least two seconds (sec), was automatically recorded using Startlefreezing software (Panlab Harvard Apparatus). Freezing response is a reliable measure of conditioned fear in rodents and is the behavioural response of the associative learning process produced by pairing a neutral CS with an aversive US. The percentage of time spent freezing was calculated by dividing the absolute freezing time by the total time analyzed in each session, averaged per genotype.

3.3.1.1 Pure contextual fear conditioning

The pure contextual fear-conditioning paradigm was performed by presenting random aversive foot shocks (US) in an initially neutral context (CS) to elicit a freezing response. On day 1, animals were placed in the testing chamber for a three minutes (min) habituation session (baseline). Twenty-four hours later (day 2), mice were trained in the same chamber in a single five min session with two min exploration, followed by five trials with US presentation (foot shock: 2 sec, 0.2 mA), separated by a variable CS presentation interval (inter-trial interval ITI, 15 - 60 sec); mice remained in the chamber for 30 sec after the last US presentation. Freezing behaviour was measured during 15 sec after each shock, for constructing the learning curve. Total seconds freezing during the CS presentations in the inter-trial intervals (ITI) separating each shock, were scored as a measure of explicit conditioning for each mouse, and this number was expressed as a percentage of the total CS exposition time. Twenty-four hours (day 3) and one week (day 9) after training mice were tested for contextual fear memory by measuring freezing time in the same training chamber in a two min testing session.

A total 22 WT and 19 Tg*NTRK3* animals were used in the experiment. Data were analyzed using repeated measures two-way analysis of variance (2-way ANOVA) with genotype and trial as factors (training session) and Student's t-test to compare test effects (test sessions).

3.3.1.2 Trace fear conditioning

In this behavioural procedure mice were trained to associate the neutral stimulus (a tone, CS) with an aversive stimulus (electric foot shock, US), separated by a trace time interval. On day 1, animals were placed in the testing chamber for a three min habituation session and basal freezing levels were registered. On the second day, mice were trained in the same chamber in a seven min session composed of two min exploration followed by five CS-trace-US presentations (CS: 10 sec, 100 dB, 2000 Hz; trace: 18 sec interval; US: 2 sec, 0.2 mA), separated by a variable ITI (15 to 60 sec). Freezing behaviour was measured during the 18 sec of each trace interval to generate a learning curve. Twenty-four hours (day 3) and one week (day 9) after the training session, mice were tested for cue-temporal fear memory in the same chamber but with different floor texture, walls color and pattern, odor and light intensity (new context), following the same training protocol but without US presentation. Freezing time was scored during 20 sec after the first CS presentation (the equivalent trace-US time interval of the training session).

A total 19 WT and 21 Tg*NTRK3* were used in this experiment and data were analyzed using Student's t-test (test days) and repeated measures 2-way ANOVA with genotype and trial as factors (training session).

3.3.1.3 Delay fear conditioning

In the delay fear-conditioning paradigm mice were trained to associate a sound (CS: 30 sec, 100 dB, 2000 Hz) with a contiguous aversive foot shock (US: 2 sec, 0.2 mA). The protocol consisted in a habituation session on day 1, in which animals were placed in the conditioning box for three min and basal freezing behaviour was registered. On day 2, mice were returned to the box for the training session composed of a four min exploration phase followed by a 30 sec CS presentation that was paired with a two sec US, administered at the end of the CS. Mice were allowed to recover for 30 additional sec before returning to the home cage. On day 3, fear memory was measured in the same box in a test session composed of two min exploration followed by three min CS presentation, during which freezing behaviour was registered.

A total 9 WT and 7 Tg*NTRK3* mice were used in the experiment and data were analyzed with Student's t-test.

3.3.2 Pure contextual fear extinction, reinstatement and contextual generalization

Extinction

Extinction was attained by unpairing the CS and US stimuli. WT and Tg*NTRK3* mice were trained in the pure contextual fear conditioning paradigm (see above 3.3.1.1) and twenty-four hours after conditioning were presented to the conditioned context (CS) without reinforcement by the US. The extinction phase consisted of one single session of six trials (E1-E6), each lasting two min and separated from the next by one-hour interval. The following day (day 4) mice were tested for fear memory extinction in the same context for two min.

Reinstatement

To test for reinstatement of the extinguished memory Tg*NTRK3* and WT mice received a single foot shock two min after the extinction test and freezing time was recorded within two min after the US presentation.

Contextual generalization

Twenty-four hours after reinstatement, mice were tested for contextual generalization in the same chamber but with different floor texture, wall color and pattern, odor and light intensity (new context), and freezing was measured for two min.

A total of 31 WT and 28 Tg*NTRK3* were used for these experiments. A group of animals was sacrificed after extinction training and another after extinction test for future immunohistochemical analysis. Thereafter, 21 WT and 19 Tg*NTRK3* were tested for fear memory extinction and 16 WT and 14 Tg*NTRK3* were used for both reinstatement and contextual generalization. Data were analyzed using repeated measures 2-way ANOVA with genotype and trial as factors (extinction training), Student's t-test (test session) and 2-way ANOVA with genotype and test as factors as compared to the extinction memory test (reinstatement and contextual generalization).

3.4 Histology

To collect brain slices, animals were anesthetized (mixture of 0.1 mg/Kg ketamine and 1 mg/Kg medetomidine) and intracardially perfused with phosphate buffered saline (PBS, 0.1M pH 7.6), followed by fixation with 4% paraformaldehyde (PFA) in PBS. Brains were removed and kept in 4% PFA, twenty-four hours at 4 ºC for post-fixation and then in a solution of 30% sucrose in PBS for two days at 4 ºC. Six series of 40 µm free floating coronal sections (each 240 µm apart) were obtained using a Vibrotome (VT1000S, Leica Microsystems, Wetzlar, Germany) and stored at -20 ºC in cryoprotector (30% ethylenoglycol, 30% Glycerol, 40% PBS) until used. Before immunostaining, sections were extensively washed with PBS to eliminate cryoprotector traces.

3.4.1 Immunohistochemistry

3.4.1.1 Immunostaining

GAD 65/67

Free floating brain sections were permeabilized with 0.3% Triton X-100 in PBS 0.1M pH 7.6, for 30 min at room temperature (RT). Antigen retrieval was performed by incubating the slices with a 50% formamide / 50% 2x saline-sodium citrate (SSC) solution for two hours at 65 ºC, passed to a 2N hydrochloric acid (HCl) solution for 30 min at 37 ºC and finally incubated in borate buffer 0.1 M pH 8.5 for three min at RT. Endogenous peroxidases were inactivated with 3% hydrogen peroxide (H_2O_2) in PBS, for 15 min at RT and protected from light. Non-specific binding sites were blocked with 3% bovine serum albumin (BSA), 0.3% Triton X-100 in PBS for one hour at RT. Sections were incubated with primary antibody rabbit anti-Gad 65/67, overnight at 4 ºC (1:250, AB1511, Chemicon, Temecula, CA, USA). Detection was performed by incubation with biotinylated secondary antibody goat anti-rabbit (1:200, BA-1000, Vector Laboratories, CA, USA) for one hour at RT. Staining was developed using the avidin-biotin-peroxidase complex (Vectastain ABC kit Vector Laboratories) and DAB as a chromogen. Counterstaining of nuclei was performed with crystal violet.

cFOS

For cFOS immunostaining, brain sections were incubated with a solution of 10% methanol (MetOH) / 3% H_2O_2 in PBS for 30 min at RT (protected from light) to block endogenous peroxides. Slices were permeabilized with 0.2% Triton X-100 in PBS 2x 5 min incubations at RT, and blocking was performed with 10% fetal bovine serum (FBS) / 0.2% Triton X-100 in PBS for one hour at RT. Subsequently, sections were incubated overnight at 4 ºC with the primary antibody polyclonal rabbit anti-cFOS (1:1000, H-125, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Incubation with the secondary antibody biotinylated goat anti-rabbit (1:300, BA-1000, Vector Laboratories) was performed for one hour at RT. The staining was developed using the avidin-biotin-peroxidase complex (Vectastain ABC kit, Vector Laboratories) and DAB as a chromogen. Counterstaining of nuclei was performed with crystal violet.

3.4.1.2 Image acquisition and quantification analysis

Quantification of positive nuclei was performed manually in one of every sixth section (240 μm apart), covering the entire prefrontal cortical area (bregma 2.10 to 1.54), the hippocampus (dorsal hippocampus: bregma -1.34 to -2.18, ventral hippocampus: bregma -2.30 to -3.64) and amygdala (bregma -0.94 to -1.82) from each animal, and using an optical microscope (BX51, Olympus, Ballerup, Denmark) and CAST grid stereology system (Olympus). GAD65/67-positive and cFOS-positive neurons were counted in ACC, PL and IL sub-regions of the medial prefrontal cortex. In the hippocampus, the analysis was performed in the Or, Py and Rad layers of both CA1 and CA3 sub-regions and in the granular layer of the DG. In the amygdala, LA, BA, lITC, mITC, CeL and CeM were analyzed. Each region was manually drawn and results are reported as mean number of positive neurons / mm^2 ± standard error of the mean (SEM). Statistical analysis was performed using Student's t-test and 2-way ANOVA with genotype and session as factors, when two or more than two groups were compared, respectively.

3.4.2 Immunofluorescence

3.4.2.1 Immunostaining

Free floating brain sections were permeabilized with 0.3% Triton X-100 in PBS for 30 min at RT and blocked with 3% BSA / 0.3% Triton X-100 in PBS for one hour, at RT. Subsequently, sections were incubated with the appropriate combination of primary antibodies as described below.

VGLUT / VGAT

Mouse monoclonal anti-vesicular glutamate transporter 1 (VGLUT1) (1:200, clone 317G6, Synaptic Systems, Göttingen, Germany) and guinea pig polyclonal anti-vesicular GABA transporter (VGAT) (1:200, cytoplasmatic domain, Synaptic Systems) antibodies were incubated overnight, at 4 ºC. The following day, slices were incubated with the corresponding secondary antibodies for fluorescence detection, alexa fluor 488 goat anti-mouse IgG and alexa fluor 555 goat anti-guinea pig IgG, (1:1000, Invitrogen, Oregon, USA) for one hour at RT and protected from light.

PSD95 / VGLUT

Mouse monoclonal anti-VGLUT1 (1:200, Clone 317G6, Synaptic Systems) and rabbit polyclonal anti-postsynaptic density 95 (PSD95) (1:250, ab18258, Abcam, Cambridge, UK) antibodies were incubated overnight, at 4 ºC. The following day, slices were incubated with the corresponding secondary fluorescent antibodies for detection, alexa fluor 555 goat anti-mouse IgG and alexa fluor 488 goat anti-rabbit IgG, (1:1000, Invitrogen) for one hour at RT and protected from light.

cFOS

Rabbit polyclonal anti-cFOS antibody (1:500, H-125, Santa Cruz Biotechnology) was incubated overnight at 4 ºC. The following day slices were incubated with the secondary antibody alexa fluor 594 goat anti-rabbit IgG (1:1000 Invitrogen) for one hour at RT protected from light.

At the end, sections were mounted and nuclei stained on glass slides with Vectashield with DAPI (Vector Laboratories).

3.4.2.2 Image acquisition and quantification analysis

The analysis of the VGLUT1 / VGAT and PSD95 / VGLUT1 was performed in one of every sixth brain section, covering the entire dorsal and ventral hippocampus from each animal. Images were captured in the *Rad* of CA1 and CA3 sub-regions of both right and left hippocampus (Bregma -1.34 / -2.18, dorsal; -2.30 / -3.64, ventral) with a confocal microscope (TCS SPE, Leica Microsystem) using a 63x objective and 5x magnification. For each sub-region, all images of the same experimental group were captured with identical confocal settings for laser' power, gain and offset levels.

For quantification analysis of VGLUT1 and VGAT staining, images were imported into IMAGEJ 1.42l (Macbiophotonics, Hamilton, ON, Canada). First, the negative control background intensity was subtracted from each channel of each image, converted into binary data and threshold to outline immunopositive puncta. The number and size of VGLUT1 and VGAT puncta per field were quantified using the *'analyze particle'* function of the software. Data were presented as VGLUT / VGAT ratio and reported separately for dorsal and ventral hippocampus as mean ± SEM (WT, n = 7 and Tg*NTRK3*, n = 9). Data were analyzed with Student's t-test.

For co-localization analysis of PSD95 / VGLUT1 puncta, images were imported into IMAGEJ 1.42l (Macbiophotonics). After, the negative control background intensity was subtracted to each channel of each image, converted into binary data and threshold to outline immunopositive puncta. Superimposing threshold images of each channel was performed with the 'co-localization highlighter' plugin of the software. The number of PSD95 / VGLUT1 double positive puncta was quantified and averaged per genotype (WT, n = 8 and Tg*NTRK3*, n = 8). Data were analyzed with Student's t-test.

Analysis of Thy1-YFP/ cFos double positive neurons was performed in one of every sixth section of each animal, covering the entire prefrontal cortex (Bregma 2.10 / 1.54), hippocampus (Bregma -1.34 / -2.18, dorsal; -2.30 / -3.64, ventral) and amygdala (Bregma -0.94 / -1.82) brain regions (WT-FC, n = 5 and Tg*NTRK3*-FC, n = 5; WT-FExt, n = 5 and Tg*NTRK3*-FExt, n = 5). For each section, images were captured with a confocal microscope (TCS SP5, Leica Microsystems) using a 40x objective. Ten µm z-stacks were captured including the following sub-regions of each region of interest: for the hippocampus, one picture in the DG; two consecutive pictures in the central part of the CA1 *Py* region, one picture in the central part of CA3 *Py region* for the hippocampus; for the mPFC, one picture per each sub-region ACC, PL and IL; and one picture in the BLA part of the amygdala (where YFP labeling is present). A z-maximum projection of each image was given by IMAGEJ 1.42l software. The area of interest was delimited and the number of YFP / cFOS double-positive neurons was quantified manually. Results are reported as mean of double-positive neurons / mm^2 ± SEM. Data analysis was performed using Student's t-test.

3.5 Molecular analysis

3.5.1 Protein extraction

WT and Tg*NTRK3* mice were anaesthetized (a mixture of medetomidine / ketamine) and intracardially perfused with PBS. Brains were removed and the hippocampus and PFC were dissected and fast frozen in dry ice. For total protein extraction, tissue was homogenized with syringe and a 20G needle in 150 µl of lysis buffer (137 mM sodium chloride (NaCl), 20 mM Tris-HCl pH 8, 1% nonyl phenoxypolyethoxylethanol (NP40) and 10% glycerol) with protease inhibitors [1 *complete* tablet and 1 mM phenylmethylsulfonyl fluoride (PMSF) and phosphatase inhibitors (1mM sodium fluoride (NaF) and 1 mM sodium orthovanadate (Na_3VO_4)]. After homogenization, samples were vortex and kept in ice for five min (repeated three times). At the end, samples were centrifuged at 13200 rpm for 30 min at 4 °C and the supernatant collected and stored at -80 °C. Protein quantification was done with the BCA protein assay kit (Thermo Scientific, Rockford, IL, USA).

3.5.2 Western blot for TrkC neurotrophin receptors

Forty μg of total protein extract from hippocampus and PFC brain homogenates of WT and Tg*NTRK3* (n = 6 per group) were loaded in *NuPAGE* 4-12% Bis-Tris polyacrylamide gel (NP0336, Novex, Life technology, Invitrogen, Carlsbad, CA, USA) and bands were separated by electrophoresis. Proteins were transferred to nitrocellulose membrane using the *iBlot Gel Transfer Stacks* kit (IB301001, Novex,) and unspecific binding was blocked in *Odyssey blocking buffer* (927-40000, Novex). After, membranes were incubated at RT for one hour with primary goat anti-TrkC antibody (1:1000, Ref: 07-226, Upstate, Temecula, CA, USA) and for 15 min with a mouse anti-GAPDH (1:4000, Ref: MAB 374, Millipore, Temecula, CA, USA), used as a housekeeping protein. Corresponding secondary antibodies polyclonal rabbit anti-goat Alexa Fluor 680 (1:6000, Ref: A-21088, Invitrogen) and donkey anti-mouse IRDye 800 CW (1:15000, Ref: 926-32212, LI-COR) were incubated with membranes for one hour at RT. Detection was performed with ODYSSEY ® infrared scanner and bands were quantified using ODYSSEY ® software. Statistical analysis was performed using 2-way ANOVA with genotype and treatment as factors (naïve vs. FC and naïve vs. FExt) for each brain region.

3.5.3 Quantification of NT3 levels by enzyme-linked immunosorbent assay (ELISA)

Quantification of NT-3 levels was performed using the *Mouse NT3 ELISA kit* (BEK-2079, Biosensis, Thebarton, Australia). Three hundred μg of total protein extract from hippocampus and PFC brain homogenates of WT and Tg*NTRK3* (n = 5 per group) were used to detect a reliable amount of NT3. Briefly, samples (300 μg of total protein extract, in duplicate) and protein standards (mouse NT3 protein from 0 to 1000 pg/ml concentrations range) were incubated with anti-mouse NT3 primary antibody overnight at 4 °C with agitation in pre-coated 96-well microplates. The “sandwich” incubation was performed with biotinylated anti-mouse NT3 for two hours with 400 rpm agitation at RT. Development was performed using the avidin-biotin-peroxidase complex (ABC) for one hour at RT with agitation at 400 rpm and incubation with TMB color developing agent

(substrate for peroxidase reaction) for 10 min. Reaction was stopped with TMB stop solution. The amount of NT3 was quantified by reading absorbance at 450 nm on a Versa max plate reader (Molecular Devices, Sunnyvale, CA, USA).
Statistical analysis was performed using 2-way ANOVA with genotype and treatment as factors (naïve vs. FC and naïve vs. FExt) for each brain region.

3.6 *In vivo* pharmacology

3.6.1 Systemic ifenprodil and tiagabine administration

WT and Tg*NTRK3* mice were submitted to the pure contextual fear-conditioning paradigm (see section 3.3.1.1). Immediately after the training session, mice were injected intraperitoneally (i.p.) with either ifenprodil 1 mg/Kg (Sigma, Saint Louis, MI, USA), a NMDA receptor 2B (NR2B) antagonist or tiagabine 10 mg/Kg (Sigma), a GABA reuptake inhibitor. Saline injected mice were used as controls. Twenty-four hours after drug administration all mice were tested for contextual fear memory.
The doses of Ifenprodil and tiagabine used in these experiments were determined by performing a dose-response curve in separate groups of WT animals. For ifenprodil we used 0.5, 1.0, 5.0 and 10.0 mg/Kg (n = 10 to 12 animals per group) and for tiagabine we used 2.5. 5.0 and 10.0 mg/Kg (n = 5 to 8 animals per group) and compared the treated animals to saline injected controls (n = 5 to 8). Statistical analysis was conducted using repeated measures 1-way ANOVA with treatment as factor (training session) and 1-way ANOVA with treatment as factor (test and re-test).

For the pure contextual fear-conditioning experiments WT and Tg*NTRK3* were distributed in drug or saline treated groups [Ifenprodil: WT-saline (n = 9), WT-1mg/Kg ifenprodil (n = 10), Tg*NTRK3*-saline (n = 10) and Tg*NTRK3*-1mg/Kg ifenprodil (n = 11); Tiagabine: WT-saline (n = 7), WT-10mg/Kg tiagabine (n = 8), Tg*NTRK3*-saline (n = 9) and Tg*NTRK3*-10mg/Kg tiagabine (n = 10)]. Statistical analysis was conducted using repeated measure 2-way ANOVA with genotype and treatment as between-subject factors and post-shocks (ITI) freezing as

within-subject factors (training) and 2-way ANOVA with genotype and treatment as factors (test and re-test).

3.6.2 Mouse brain stereotaxic surgery

Animals were injected subcutaneously (s.c.) with 0.05 mg/Kg buprenorfine and 15 min later were deeply anesthetized with a mixture of medetomidine and ketamine (i.p., 1 mg/Kg and 75 mg/Kg, respectively). After complete loss of reflexes, the head was fixed in a stereotaxic apparatus (Just for Mice™ Standard Stereotaxic Instrument, Harvard Apparatus, Holliston, MA, USA). After ethanol sterilization of the surface, an incision was made in the skin along the midline of the head. Two holes were opened in the skull, using a driller, corresponding to regions of interest according to stereotaxic coordinates (Paxinos 2001): ventral hippocampus, antero-posterior (AP) = - 3.0, medio-lateral (ML) = ± 3.0, dorso-ventral (DV) = - 2.3; PFC, AP = + 2.0, ML = ± 0.50, DV = - 1.2.

Bilateral cannulae (0.5 outer / 0.25 inner diameters, AISI 304 Unimed, Lausanne, Switzerland) were implanted into the brain and fixed with dental cement (Simplex Rapid, Kemdent, UK). Skin was closed using histologic glue in the two fronto-caudal opposite limits. Animals were awaked with antisedan (i.p., 2 mg/Kg) and eye dryness was avoided by local application of ophthalmic ointment. The following three days after surgery, mice were administered buprenorfine anaelgesia (i.p., 0.05 mg/Kg) and checked for general health status. Behavioural procedures were performed at least seven days after surgery.

3.6.3 Intra-hippocampal tiagabine administration

WT and Tg*NTRK3* mice were trained in the pure contextual fear-conditioning and, immediately after training, animals were infused with tiagabine (0.5 µl per side from a 2 mg/ml solution, SLM0035, Sigma-Aldrich) or saline. The infusion was performed in manually immobilized animals through an internal micro-cannula (0.2 outer/0.09 inner diameters, AISI 316L Unimed), connected with a 15 cm long PVC (polyvinyl chloride) tube (0.25 inner diameter, F117952, Gilson,

Middleton, WI, USA) to a 5 μL syringe (Model 75, Hamilton, Reno, NE, USA). The internal cannula was exiting from the implanted cannula for one mm and the infusion was performed with a rate of 250 nL/min. After the injection, animals were returned to their home cage and twenty-four hours and one week later were tested for contextual fear memory (see section 3.3.1.1). A total of n = 5 WT-saline, n = 5 WT-1 μg/side tiagabine, n = 5 Tg*NTRK3*-saline and n = 6 Tg*NTRK3*-1 μg/side tiagabine mice were used in the experiment. Data were analyzed using repeated measure 2-way ANOVA with genotype and treatment as between-subject factors and post-shocks (ITI) freezing as within-subject factors (training) and 2-way ANOVA with genotype and treatment as factors (test and re-test).

3.6.4 Intra-medial PFC NT3 administration

Animals were fear conditioned using the pure contextual fear-conditioning paradigm (see section 3.3.1.1) and submitted to the fear extinction training protocol (see section 3.3.2). One hour after extinction training, recombinant human NT3 (rhNT3, 0.75 μl per side from a 1 mg/ml solution, C079, Novoprotein, Shanghai, China) or saline were infused in the mPFC. The infusion was performed in manually immobilized animals using a micro-cannula (0.2 outer / 0.09 inner diameters, AISI 316L, Unimed), connected through a 15 cm long PVC tube (0.25 inner diameter, F117952, Gilson) to a 5 μL syringe (Model 75, Hamilton). The internal cannula was exiting from the implanted for one mm and the infusion was performed with a rate of 250 nL/min. After the infusion, animals were returned to their home cage. The day after, contextual fear extinction memory was tested in WT-saline (n = 8), WT-0.75 μg/side NT3 (n = 10), Tg*NTRK3*-saline (n = 7) and Tg*NTRK3*-0.75 μg/side NT3 (n = 9). Data were analyzed with repeated measure 2-way ANOVA with genotype and treatment as between-subject factors and post-shocks (ITI) freezing as within-subject factors (training). Extinction was analyzed with repeated measure 2-way ANOVA with genotype and treatment as between-subject factors and extinction trials (E1-E6) freezing as within-subject factors (extinction training) and 2-way ANOVA with genotype and treatment as factors (extinction test).

RESULTS

4. RESULTS

4.1 Characterization of fear learning and memory in Tg*NTRK3* mice

The first question we addressed in this project was to what extent fear learning and retention are altered in the Tg*NTRK3* mice, as it occurs in PAND patients. To this end, we used different fear conditioning behavioural tests that reflect the function of different brain sub-circuits. These paradigms are based on Pavlov's assumptions on conditioned learning in which he postulated that animals associate a neutral CS with an aversive US (foot shock) to generate a conditioned fear response during subsequent re-exposures to the CS, without reinforcement by the US.

4.1.1 Contextual fear learning and memory in Tg*NTRK3* mice

To address the involvement of the hippocampus and context in fear learning in our PAND model, we used the pure contextual fear-conditioning paradigm (Figure 1A), in which the CS is a specific context. In the acquisition session of this paradigm, both WT and Tg*NTRK3* mice showed similar low basal freezing level (before the first US presentation) (Figure 1C) and similar increase of the percentage of time freezing along the session with no statistically significant different learning curves (Figure 1B, repeated measures 2-way ANOVA, genotype effect p = 0.2 and trial effect p = 3.2E-13). Twenty-four hours after the training session, contextual fear memory was tested. Tg*NTRK3* mice show enhanced freezing response as compared to WT animals (Figure 1C, Student's t-test p = 0.014), which was maintained one week later in the re-test session (Figure 1C, Student's t-test, p = 0.055). The encoding and expression of contextual fear memory is highly dependent on the hippocampus, thus these results indicate an enhanced and sustained hippocampal-dependent fear memory in Tg*NTRK3* mice.

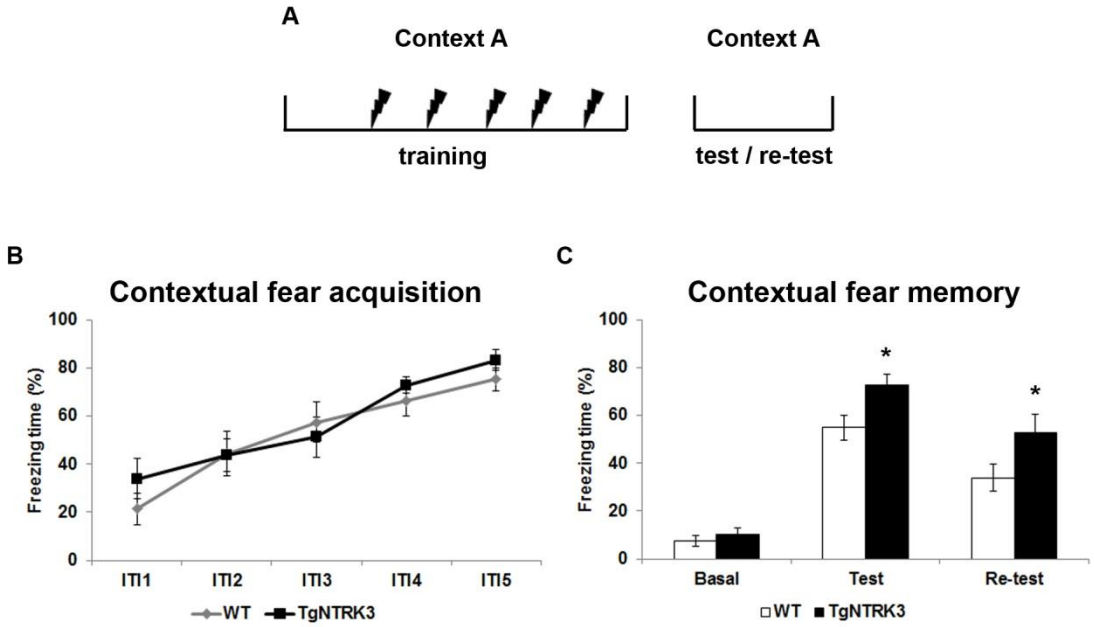

Figure 1: Performance of WT and Tg*NTRK3* mice in the pure contextual fear conditioning paradigm. (A) Scheme representing the pure contextual fear conditioning paradigm. (B) Freezing response exhibited by WT (n = 22) and Tg*NTRK3* (n = 19) mice during the acquisition session. Freezing levels were measured during exposition to CS (context), in the inter-trial intervals (ITI) separating each shock, as a measure of explicit conditioning. (C) Freezing response presented by WT and Tg*NTRK3* mice in the two min context exposure in the test and re-test sessions. Statistical analysis was performed using repeated measure 2-way ANOVA (acquisition) and Student's t-test (test and re-test), * $p < 0.05$.

4.1.2 Temporal fear learning and memory in Tg*NTRK3* mice

In the trace fear-conditioning paradigm (Figure 2A), an interval of time (trace) separates the CS (auditory tone) from the US (foot shock), creating a temporal association between the two stimuli that is, in the short term, encoded by the hippocampus. During the acquisition phase Tg*NTRK3* mice and WT littermates, showed similar levels of basal freezing (before the first CS-US) (Figure 2C) and similar fear encoding as shown by the learning curves (Figure 2B). In the test session, Tg*NTRK3* mice showed enhanced temporal fear memory (Student's t-test, $p = 0.023$), which was not maintained during the re-test session (Figure 2C). These results show that when the hippocampal function is required, such as in the test session of temporal fear memory, Tg*NTRK3* are more efficiently consolidating fear-related information than WT animals.

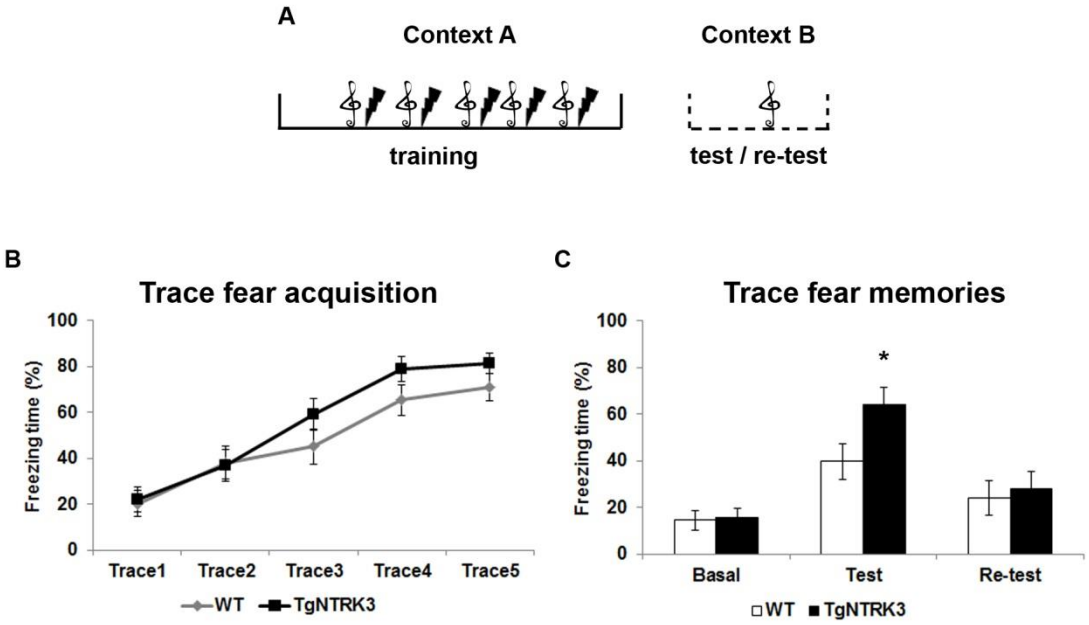

Figure 2: Performance of WT and Tg*NTRK3* mice in the trace fear-conditioning paradigm. (A) Schematic representation of trace fear conditioning paradigm. (B) Freezing response of WT (n = 27) and Tg*NTRK3* (n = 29) mice during the trace of each CS-US presentation in the training session. (C) Freezing response of WT and Tg*NTRK3* mice in the equivalent trace following the CS presentation in the test and re-test sessions. Statistical analysis was performed using repeated measure 2-way ANOVA (training session) and Student's t-test (test and re-test sessions), * $p < 0.05$.

4.1.3 Delay fear memory in Tg*NTRK3* mice

In the delay fear conditioning paradigm (Figure 3A), in which the associative memory between a salient CS (tone) and an aversive US (foot shock) is tested, the generated fear memory is mainly dependent on amygdala function. The Tg*NTRK3* mice did not show a statistically significant difference in the freezing response, as compared to WT, neither in the basal freezing (before the CS-US) nor in the test session (twenty-four hours after training) (Figure 3B), revealing that fear responses are not affected in those tests that do not involve the hippocampus as a principal structure.

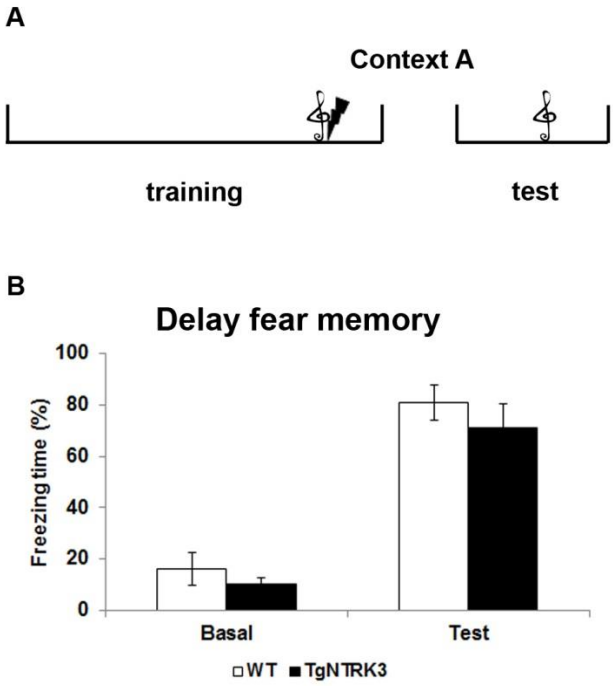

Figure 3: Performance of WT and Tg*NTRK3* mice in the delay fear conditioning paradigm. (A) Schematic representation of the delay fear conditioning paradigm. (B) Freezing response of WT (n = 9) and Tg*NTRK3* (n = 7) mice during the test session. Statistical analysis was performed using the Student's t-test.

All together these three fear conditioning experiments revealed specific hippocampus-dependent enhanced fear responses in Tg*NTRK3* mice without an otherwise affected phenotype.

4.1.4 Contextual fear extinction learning and memory in Tg*NTRK3* mice

The finding that the enhanced contextual fear memory in Tg*NTRK3* is long-lasting suggests that extinction processes might also be impaired. In fact, the exaggerated fear responses PAND patients are accompanied by impairments in the extinction of these fears.

WT and Tg*NTRK3* mice were tested in the pure contextual fear extinction paradigm (Figure 4A). In this test, mice extinguish the previously learned association between the context (CS) and the US upon repeated exposures to the CS, without reinforcement by the US.

As described above, Tg*NTRK3* and WT littermates did not show statistically significant differences in their freezing levels during the acquisition session of the contextual fear paradigm (Figure 4B). In the extinction training session, Tg*NTRK3* showed higher freezing levels than WT mice (Figure 4C; repeated measures 2-way ANOVA, genotype effect p = 5.0E-05, trial effect p = 5.2E-09). Twenty-four hours post extinction-training, mice where tested for extinction memory and, again, Tg*NTRK3* showed significantly higher freezing levels than WT littermates (Figure 4D, Student's t-test p = 0.001).

In the reinstatement session, in which the original fear memory is re-activated by the presentation of a single US, both genotypes show a statistically significant increase in the freezing response, as compared to the extinction freezing levels (Figure 4D, 2-way ANOVA, test effect p = 3.8E-06), with Tg*NTRK3* maintaining the freezing response higher than WT (Figure 4D, 2-way ANOVA, genotype effect 6.9E-05).

Finally, we tested the animals for contextual generalization, by measuring freezing levels in a novel context one day after reinstatement. Both WT and Tg*NTRK3* mice show reduced freezing levels as compared to extinction freezing response (Figure 4D, genotype x test effect p = 0.083, *post hoc* Bonferroni, contextual generalization: WT vs. Tg*NTRK3* p = 0.15, extinction: WT vs. Tg*NTRK3* p = 4.4E-05).

In summary, the pure contextual fear extinction paradigm reveals a marked impairment in Tg*NTRK3* ability to extinguish acquired contextual fear, which is not generalized to other contexts, suggesting an altered neural functioning in the hippocampal – amygdala – prefrontal cortex loop.

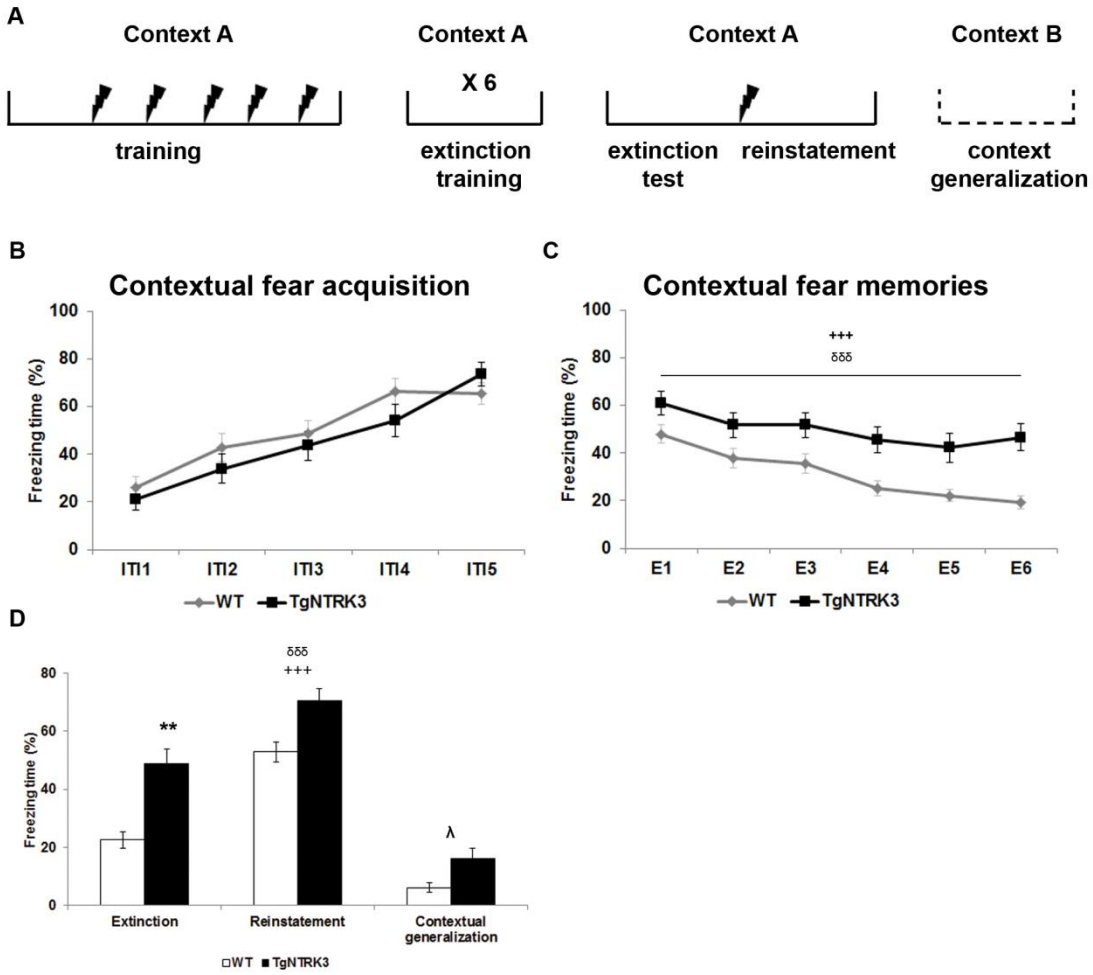

Figure 4: Performance of WT and Tg*NTRK3* mice in the pure contextual fear extinction paradigm. (A) Schematic representation of contextual fear conditioning, extinction, reinstatement and contextual generalization behavioural paradigms. Freezing response of WT (n = 26) and Tg*NTRK3* (n = 22) mice during (B) training and (C) extinction training sessions. (D) Freezing response in the extinction test (WT n = 21 and Tg*NTRK3* n = 19), reinstatement and contextual generalization sessions (WT n = 16 and Tg*NTRK3* n = 14). Statistical analysis was performed using repeated measures 2-way ANOVA for genotype and trial (fear conditioning and extinction training), Student's t-test (extinction test) and 2-way ANOVA with genotype and test as factors (reinstatement and contextual generalization, as compared to extinction), followed by *post hoc* Bonferroni analysis. Extinction training: δ genotype effect; + trial effect, $^{\delta\ \delta\ \delta/+++}$ $p < 0.001$. Extinction test: Student's t-test, ** $p < 0.01$. Reinstatement and Contextual generalization: δ genotype effect; + test effect, λ genotype x test interaction, $^{\delta\ \delta\ \delta/+++}$ $p < 0.001$, $^{\lambda}$ $p < 0.05$.

4.2 Fear brain circuit activation pattern in Tg*NTRK3* mice

The data obtained in the behavioural experiments suggested that Tg*NTRK3* mice might present phenotypic traits in brain regions implicated in the encoding and / or storage of fear information.

Concretely, the hippocampus, strongly involved in the processing of contextual information, the amygdala, the core brain area in fear processing and the medial prefrontal cortex, required mostly for extinction learning (Figure 5). C-FOS activation follows behavioral training and correlates with an acquisition of the behaviorally measured response, i.e. with fear learning. Thus we characterized the activation pattern of the hippocampus, amygdala and prefrontal cortex in WT and Tg*NTRK3* mice in (1) naïve conditions, (2) one hour after contextual fear training and (3) one hour after contextual fear extinction training, by counting the number of cFOS-positive (activated) neurons in different sub-regions.

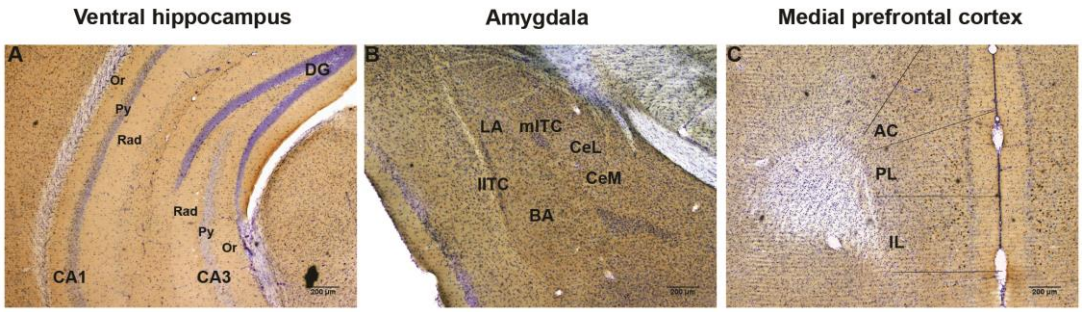

Figure 5: Representative photomicrographs of c-FOS neuronal activation in the hippocampus, amygdala and mPFC of WT and Tg*NTRK3* mice. Low magnification representative pictures of the (A) ventral hippocampus, (B) amygdala and (C) medial prefrontal cortex of brain slices immunostained for c-Fos, depicting layers and subregion subdivisions. Or, *stratum oriens*; py, *stratum pyramidale*; rad, *stratum radiatum*; DG, dentate gyrus; CA1-CA3, *Cornu Ammonis*; LA, lateral amygdala; lITC, lateral intercalated cell cluster; BA, basal amygdala; mITC, medial intercalated cell cluster; CeL, centro-lateral amygdala; CeM, centro-medial amygdala; AC, anterior cingulum, PL, pre-limbic; IL, infra-limbic.

4.2.1 Neuronal activation pattern upon contextual fear conditioning in Tg*NTRK3* mice

In the CA1py and CA3py layers of the ventral hippocampus, the encoding of fear information during the fear conditioning (FC) training session led to an increase of the number of c-FOS positive neurons both in WT and Tg*NTRK3* mice, as compared to naïve condition (Figure 5A, 6AD, 7B ventral CA1py p = 8.2E-05 and CA3py p = 4.2E-05). Interestingly, Tg*NTRK3* showed a significantly higher activation of the CA1py neurons as compared to WT (genotype x FC interaction p = 0.048; Bonferroni: WT-naïve vs. WT-FC p = 0.052, Tg*NTRK3*-naïve vs. Tg*NTRK3*-FC p = 7.9E-05, Tg*NTRK3*-FC vs. WT-FC p = 0.013). In CA3py, a significant genotype effect was found (p = 0.024) towards an increased number of c-FOS positive cells in Tg*NTRK3* mice. No differences between genotypes or treatment effects were observed in any other hippocampal layer (DG, CA1/3Or, CA1/3Rad; Figure 7B, C).

In the dorsal hippocampus, FC produced a similar increase in both genotypes of the activation of CA1py and CA3py layers (Figure 7D, FC effect: dorsal CA1py p = 0.017 and CA3py p = 7.7E-04). Interestingly, in the CA3Or, Tg*NTRK3* showed an increased number of c-FOS positive cells in naïve condition, as compared to WT, which was reduced by FC (Figure 7E, genotype x FC interaction p = 0.008; Bonferroni: WT-naïve vs. Tg*NTRK3*-naive p = 0.019, Tg*NTRK3*-naïve vs. Tg*NTRK3*-FC p = 0.015).

In the amygdala we quantified the number of activated neurons in all the nuclei, involved in fear learning. No significant differences were found between genotype or upon exposure to FC in neither the LA nor the BA (Figure 5B, 7F), assumed to be one of the entry routes into the amygdala circuit. However, in the lITC, that modulates the BLA amygdala, naïve Tg*NTRK3* showed an increased number of c-FOS positive neurons compared to the WT controls, which were normalized upon FC in Tg*NTRK3* mice (Figure 7F, genotype x FC interaction p = 0.025, Bonferroni: WT-naïve vs. Tg*NTRK3*-naïve p = 0.023, Tg*NTRK3*-naïve vs. Tg*NTRK3*-FC p = 0.039). In the CeL nucleus, known to contain neurons inhibiting the output CeM cells, the number of c-FOS positive cells in both WT

and Tg*NTRK3* was reduced after fear conditioning (Figure 7G; FC effect p = 0.006) and in the CeM, that orchestrates the final fear response, a significant genotype x FC interaction was detected (Figure 6E-H, 7G, p = 0.034). *Post hoc* comparisons revealed that in naïve condition the CeM of Tg*NTRK3* mice was already more activated than in WT and fear conditioning led to an even higher activation in Tg*NTRK3*, but not in WT animals (WT-naïve vs. Tg*NTRK3*-naïve p = 0.003, WT-FC vs. Tg*NTRK3*-FC p = 2.2E-06, Tg*NTRK3*-naïve vs. Tg*NTRK3*-FC p = 4.0E-04). In the mITC, mostly containing interneurons operating a local inhibitory control in the central sub-circuit, we observed again increased activation in the naïve condition in transgenic mice, and a differential activation pattern, as compared to WT: here FC increased the number of c-FOS cells in WT mice, but not in Tg*NTRK3* (Figure 7G, genotype x FC interaction p = 0.014; Bonferroni, WT-naïve vs. WT-FC p = 0.049, WT-FC vs. Tg*NTRK3*-FC p = 0.028) suggesting an impaired local inhibitory circuit activation within the amygdala loop.

The mPFC plays an important modulatory role of the activation of the amygdala, mainly to inhibit or reduce fear responses. Here, fear conditioning did not change the number of c-FOS positive neurons in the PL region of WT or Tg*NTRK3* mice, as compared to the naïve condition (Figure 5C, 7H). However, in the AC and IL regions both genotypes showed a differential pattern of activation, as fear conditioning reduced the number of c-FOS positive cells in Tg*NTRK3*, but not in WT littermates (Figure 6I-N, 7H, AC: genotype x FC interaction p = 0.096, Tg*NTRK3*-naïve vs. Tg*NTRK3*-FC p = 0.029; IL: genotype x FC interaction p = 0.048, Tg*NTRK3*-naïve vs. Tg*NTRK3*-FC p = 1.2E-04).
To sum up, in Tg*NTRK3* mice the hyperactivation of hippocampal CA3 and CA1 pyramidal layers, together with a hypoactivation of the mPFC, mainly the IL portion, could explain the overall hyperactivation of the CeM nucleus of the amygdala, lacking an adequate local inhibitory control mediated by the mITC cluster. All together this analysis revealed a complex perturbed activation pattern of the hippocampal – amygdala – prefrontal cortex fear circuit, in agreement with the enhanced contextual fear memory in Tg*NTRK3* mice.

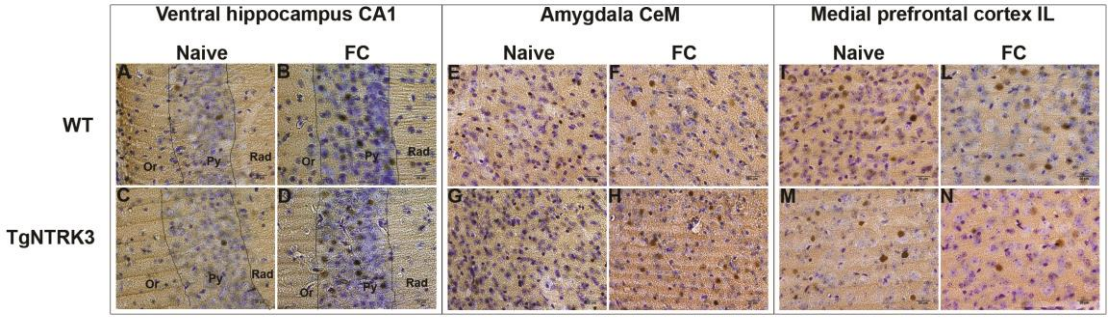

Figure 6: Representative photomicrographs of c-FOS neuronal activation in the ventral hippocampus CA1, amygdala CeM and mPFC IL regions of WT and Tg*NTRK3* mice in naïve and upon fear conditioning conditions. High magnification representative pictures of the (A-D) CA1 ventral hippocampus, (E-H) CeM amygdala and (I-N) IL medial prefrontal cortex of WT and Tg*NTRK3* mice in naïve or FC conditions. WT, wild type; Tg*NTRK3*, transgenic mice overexpressing *NTRK3*; FC, fear conditioning; Or, *stratum oriens*; py, *stratum pyramidale*; rad, *stratum radiatum*; CA1, *Cornu Ammonis 1*; CeM, centro-medial amygdala; IL, infra-limbic.

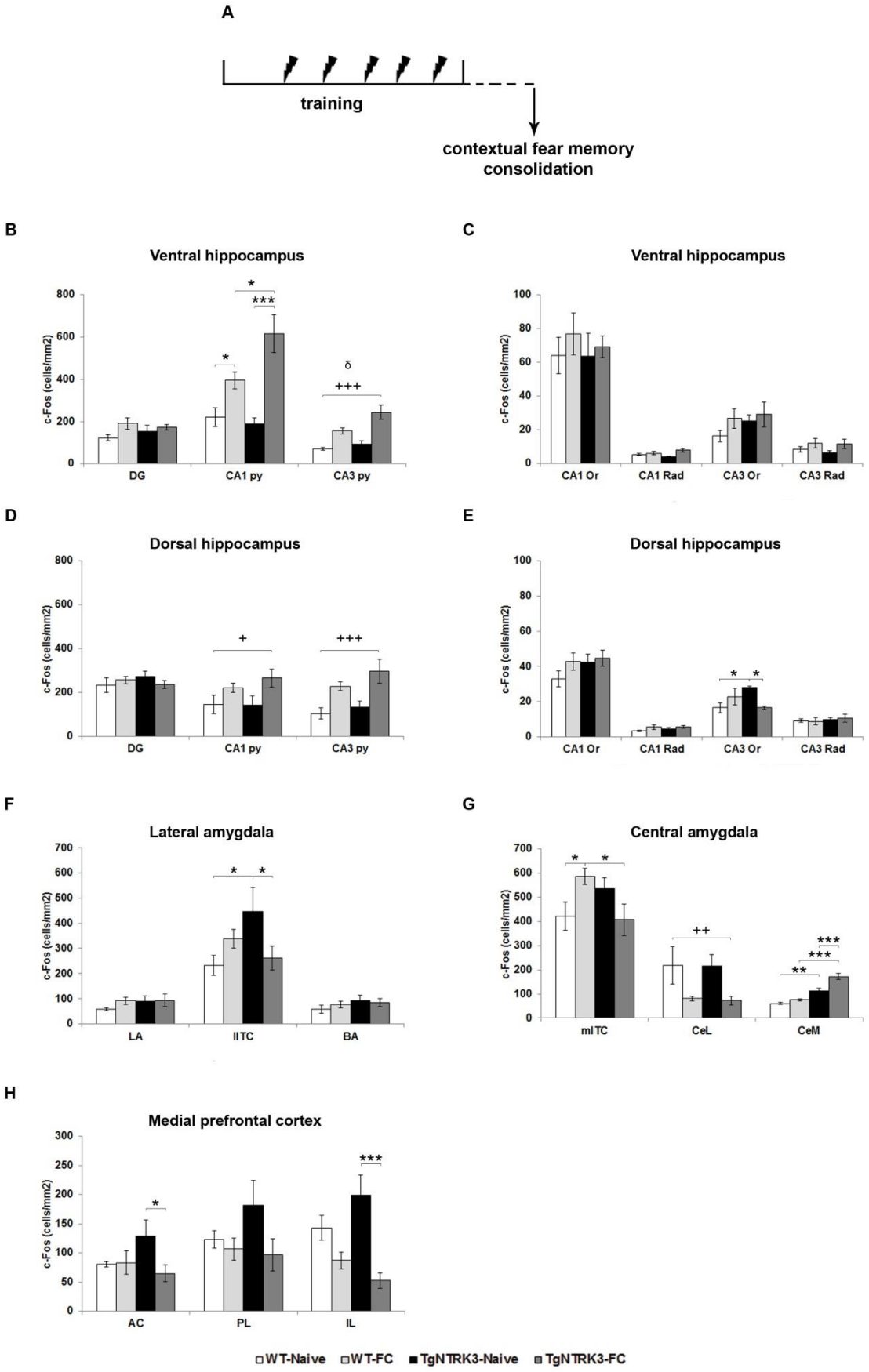
A
training
contextual fear memory consolidation
B
Ventral hippocampus
c-Fos (cells/mm2)
DG
CA1 py
CA3 py
C
Ventral hippocampus
CA1 Or
CA1 Rad
CA3 Or
CA3 Rad
D
Dorsal hippocampus
E
Dorsal hippocampus
F
Lateral amygdala
LA
lITC
BA
G
Central amygdala
mITC
CeL
CeM
H
Medial prefrontal cortex
AC
PL
IL
WT-Naive
WT-FC
TgNTRK3-Naive
TgNTRK3-FC

Figure 7: WT and Tg*NTRK3* neuronal activation pattern of amygdala – hippocampus – medial prefrontal cortex fear circuit in naïve mice and upon fear conditioning. (A) Schematic representation of pure contextual fear conditioning paradigm and time point for c-FOS analysis. (B-H) Quantification of c-FOS positive cells in naïve (n = 5 per genotype) and fear-conditioned (n = 6 per genotype) groups in (B, C) dorsal hippocampus, (D, E) ventral hippocampus, (F) lateral amygdala, (G) central amygdala and (H) medial prefrontal cortex brain regions. Statistical analysis was performed using 2-way ANOVA with genotype and state for each brain region, separately, followed by *post hoc* Bonferroni. δ, genotype effect, $^{\delta}$ $p < 0.05$; +, FC effect, $^{+}$ $p < 0.05$, $^{++}$ $p < 0.01$, $^{+++}$ $p < 0.001$; *, *post hoc* comparisons, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4.2.2 Neuronal activation pattern upon extinction of contextual fear in Tg*NTRK3* mice

In the next experiment we quantified the number of c-FOS positive neurons in WT and Tg*NTRK3* mice in naïve conditions and after contextual fear extinction training (Figure 8, 9). In the ventral hippocampus, upon extinction training Tg*NTRK3* mice, but not WT littermates, showed increased number of c-FOS positive neurons in the CA1py layer, as compared to naïve condition (Figure 8A-D, 9D; genotype x fear extinction (FExt) interaction effect p = 0.03, Bonferroni Tg*NTRK3*-naïve vs. Tg*NTRK3*-FExt p = 7.1E-04, WT-FExt vs. Tg*NTRK3*-FExt p = 0.012). In addition, FExt in WT reduced the number of c-FOS neurons in the CA1rad and CA3rad layers, known to contain local inhibitory neurons, an effect that was not observed in Tg*NTRK3* mice (Figure 9E, CA1rad: genotype x FExt interaction p = 0.011, Bonferroni WT-naïve vs. WT-FExt p = 4.0E-05, WT-FExt vs. Tg*NTRK3*-FExt p = 0.045; CA3rad: genotype x FExt interaction p = 0.028, Bonferroni WT-naïve vs. WT-FExt p = 0.004, WT-FExt vs. Tg*NTRK3*-FExt p = 0.034). A reduced number of c-FOS neurons are observed in the CA3or of both genotypes (Figure 9E, FExt effect, p = 0.01). No differences have been observed in other regions.

In the dorsal hippocampus FExt did not produce any change in the activation of DG, CA1py and CA3py layers (Figure 9B), nor in the CA1or layer (Figure 9C). However it reduced the number of c-FOS positive neurons in both genotypes in the CA1rad and CA3rad (Figure 9C, CA1rad: FExt effect p = 1.3E-04, CA3rad: FExt effect p = 1.6E-06) and in the CA3or layer, where Tg*NTRK3* showed an

increased number of c-FOS positive cells in naïve condition, FExt reduced the number of activated neurons (Figure 9C, genotype x FExt interaction p = 0.003; Bonferroni: WT-naïve vs. Tg*NTRK3*-naive p = 0.006, Tg*NTRK3*-naïve vs. Tg*NTRK3*-FExt p = 3.6E-04).

In BLA portion of the amygdala, FExt training did not produce any change in the number of c-FOS positive neurons neither in the LA nor in the BA parts in either genotype. In the lITC we found an increased number of c-FOS positive neurons in naïve Tg*NTRK3*, as compared to its respective WT controls (same naïve groups as in the FC study). However, after extinction training, the number of c-FOS positive cells in Tg*NTRK3* was reduced (Figure 9F, genotype x FExt interaction p = 0.010, Bonferroni WT-naïve vs. Tg*NTRK3*-naïve p = 0.019, Tg*NTRK3*-naïve vs. Tg*NTRK3*-FExt p = 0.03). In mITC, fear extinction reduced the number of activated neurons only in Tg*NTRK3* mice, but not in WT littermates (Figure 9G, genotype x FExt interaction p = 0.002, Bonferroni: Tg*NTRK3*-naïve vs. Tg*NTRK3*-FExt p = 1.8E-04, WT-FExt vs. Tg*NTRK3*-FExt p = 0.003). Finally FExt reduced the number of c-FOS cells in CeL in both genotypes (Figure 9G, FExt effect p = 0.015) and in CeM, Tg*NTRK3* showed increased number of c-FOS positive cells, in both naïve and after FExt conditions, as compared to WT (Figure 8E-H, 9G, WT-naïve vs. Tg*NTRK3*-naïve p = 0.001, WT-FExt vs. Tg*NTRK3*-FExt p = 1.5E-06).

In the mPFC, necessary for successful extinction of fear responses, FExt increased the number of c-FOS positive neurons in the AC of both genotypes (Figure 9H, FExt effect p = 0.03). However, in the PL and IL sub-regions only WT animals showed the expected increase of c-FOS positive neurons upon FExt, that was not observed in Tg*NTRK3* mice (Figure 8I-N, 9H, PL: genotype x FExt interaction, p = 0.061, Bonferroni WT-naïve vs. WT-FExt p = 0.014; IL: genotype x FExt interaction, p = 0.01, Bonferroni WT-naïve vs. WT-FExt p = 0.014, WT-FExt vs. Tg*NTRK3*-FExt p = 0.014).

In summary, fear extinction training in Tg*NTRK3* mice results in the hyperactivation of ventral hippocampus CA1 pyramidal neurons, and a lack of activation of the mPFC leading to impaired modulation of amygdala activation.

This perturbed fear circuit could underlie the impaired ability of Tg*NTRK3* mice to extinguish acquired contextual fear.

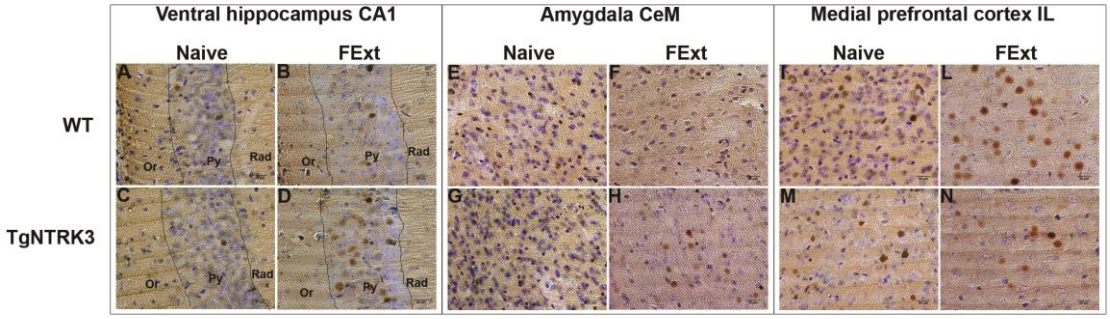

Figure 8: Representative photomicrographs of c-FOS neuronal activation in the ventral hippocampus CA1, amygdala CeM and mPFC IL regions of WT and Tg*NTRK3* mice in naïve and upon fear extinction conditions. High magnification representative pictures of the (A-D) CA1 ventral hippocampus, (E-H) CeM amygdala and (I-N) IL medial prefrontal cortex of WT and Tg*NTRK3* mice in naïve or FEXT conditions. WT, wild type; Tg*NTRK3*, transgenic mice overexpressing *NTRK3*; FExt, fear extinction; Or, *stratum oriens*; py, *stratum pyramidale*; rad, *stratum radiatum*; CA1, *Cornu Ammonis 1*; CeM, centro-medial amygdala; IL, infra-limbic.

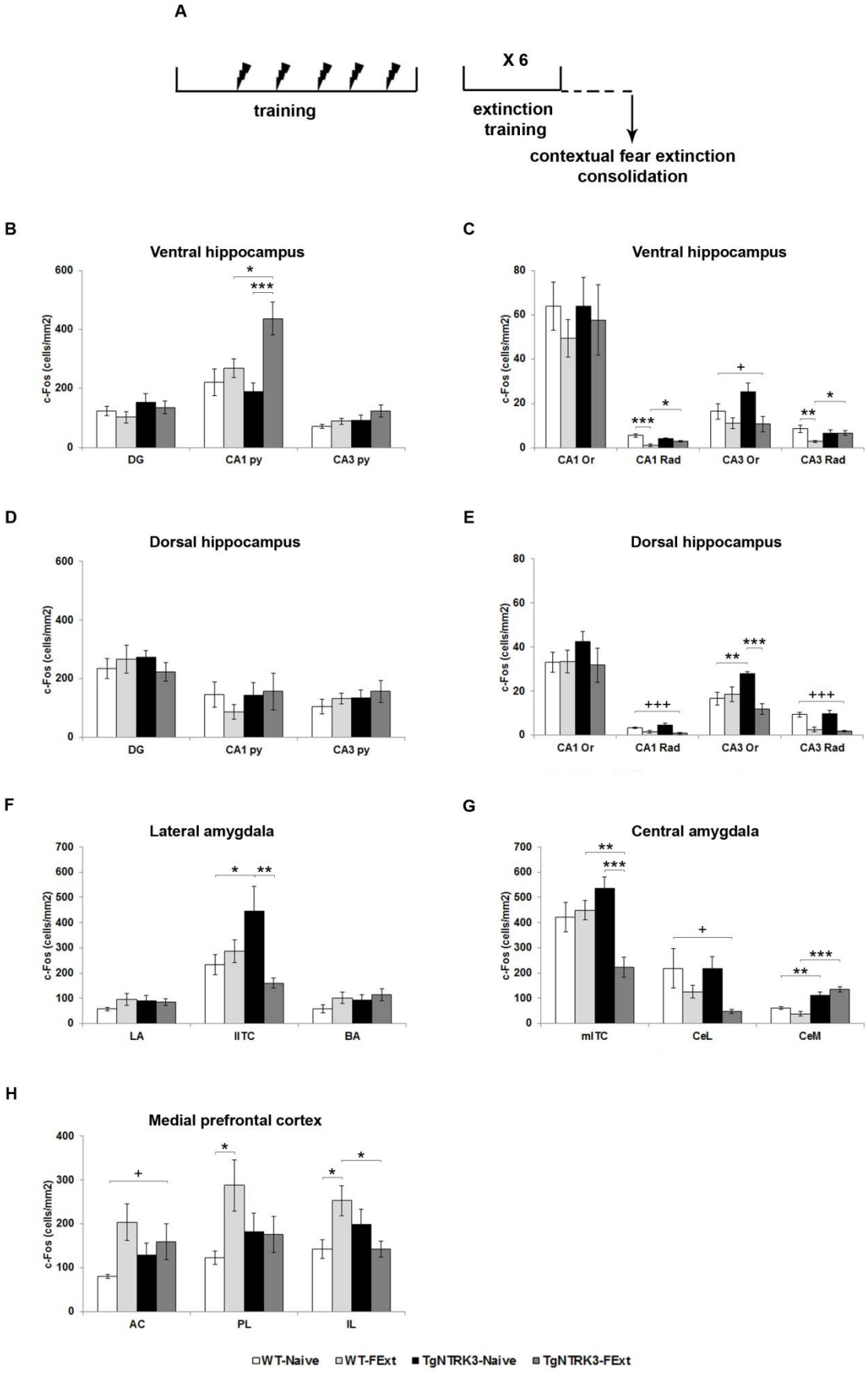
A
X 6
training
extinction training
contextual fear extinction consolidation
B
Ventral hippocampus
c-Fos (cells/mm2)
DG
CA1 py
CA3 py
C
Ventral hippocampus
CA1 Or
CA1 Rad
CA3 Or
CA3 Rad
D
Dorsal hippocampus
E
Dorsal hippocampus
F
Lateral amygdala
LA
lITC
BA
G
Central amygdala
mITC
CeL
CeM
H
Medial prefrontal cortex
AC
PL
IL
WT-Naive
WT-FExt
TgNTRK3-Naive
TgNTRK3-FExt

Figure 9: WT and Tg*NTRK3* neuronal activation pattern of amygdala – hippocampus – medial prefrontal cortex fear circuit in naïve mice and upon fear extinction. (A) Schematic representation of fear extinction (FExt) paradigm and time point for c-FOS analysis. (B-H) Quantification of c-FOS positive cells in naïve (n = 5 per genotype) and FExt (n = 5 per genotype) groups in (B, C) dorsal hippocampus, (D, E) ventral hippocampus, (F) lateral amygdala, (G) central amygdala and (H) medial prefrontal cortex brain regions. Statistical analysis was performed using 2-way ANOVA with genotype and state for each brain region, separately, followed by *post hoc* Bonferroni. +, FExt effect, $^{+}$ $p < 0.05$, $^{+++}$ $p < 0.001$; *, *post hoc* comparisons, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4.2.3 Activation pattern of hippocampal and amygdala excitatory neurons, upon contextual fear conditioning in Tg*NTRK3*

To characterize the excitatory or inhibitory nature of the c-FOS positive (activated) cells upon fear conditioning, we took advantage of the Thy1-YFP strain of mice, in which excitatory neurons express the YFP. We quantified the number of Thy1-YFP/c-FOS double positive neurons (activated excitatory neurons), one hour after contextual fear conditioning training, in the hippocampus (Figure 10A, B) and amygdala (Figure 10D, E) of Thy1-YFP/*NTRK3* and Thy1-YFP/WT double transgenic mice. PFC analysis has not been included as it plays a more important role in inhibition of fear, during extinction.

In the ventral hippocampus Thy1-YFP/*NTRK3*-FC mice showed a higher number of Thy1-YFP/c-FOS double-positive cells, as compared to Thy1-YFP/WT-FC in CA1py layer (Figure 10C, Student's t-test $p = 0.0186$) but not in the CA3py layer (Figure 10C, $p = 0.23$). No differences were found in neither the CA1py and CA3py layers of the dorsal hippocampus (data not shown), nor in the BLA amygdala (Figure 10D, E). Thus, the increased CA1py activation described in c-FOS experiments is possibly associated with higher number of activated excitatory cells of Tg*NTRK3* mice. As an intrinsic characteristic of the Thy1-YFP expression in these mice, the number of positive neurons is only a proportion of the total pyramidal neurons, in fact the total number of Thy1-YFP/c-FOS positive neurons is about the half of the number of c-FOS positive

neurons in the previous experiment (Figure 10C, 7B). Nevertheless the proportion of activated cells in ventral CA1py between WT and Tg*NTRK3* mice is maintained (1:2), thus confirming that the generated Thy1-YFP/*NTRK3* reproduced the phenotype observed in Tg*NTRK3* animals.

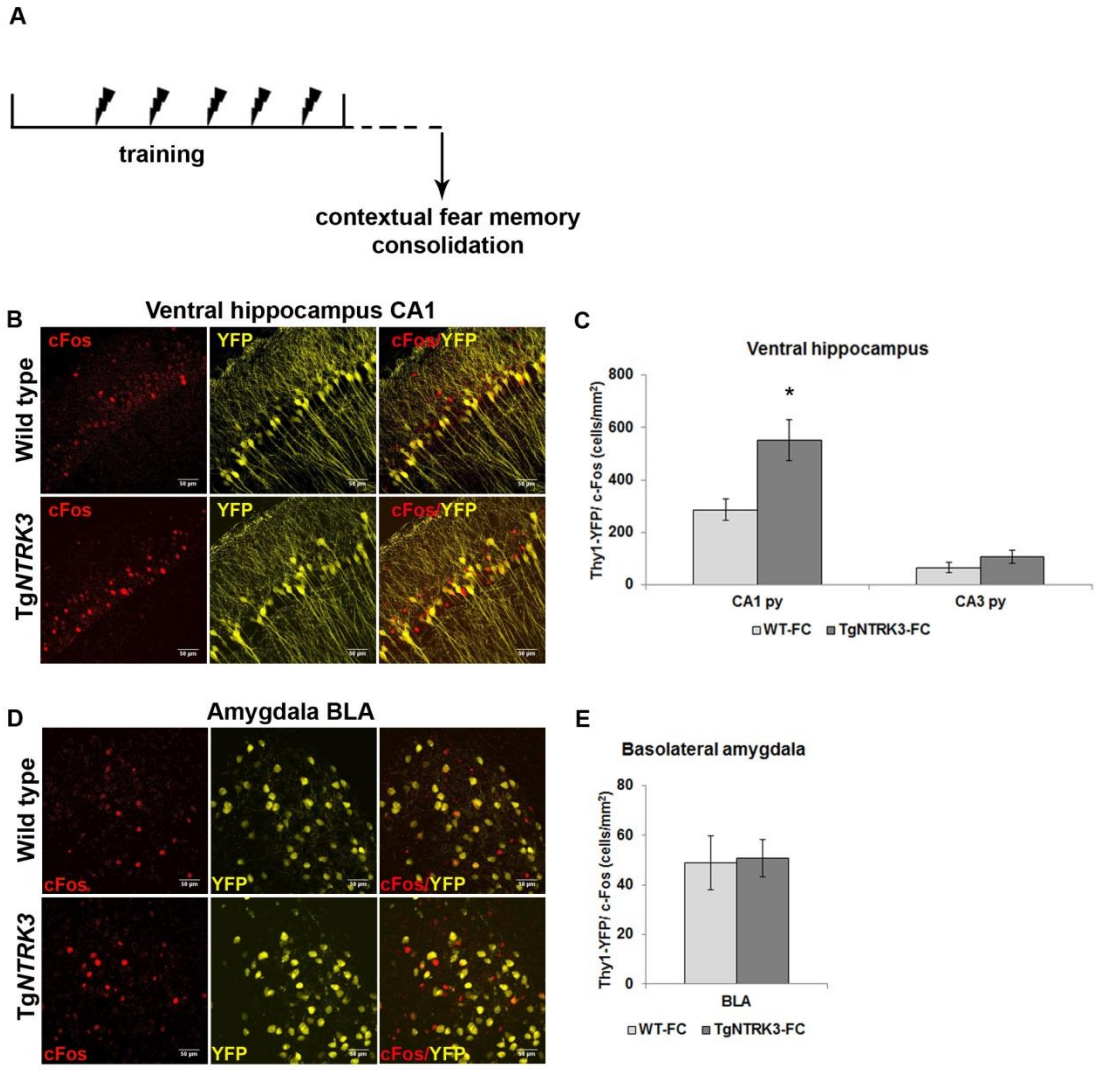

Figure 10: Activation pattern of excitatory neurons in the hippocampus and amygdala of Thy1-YFP/WT and Thy1-YFP/*NTRK3* animals after contextual fear conditioning. (A) Schematic representation of the time point during the pure contextual fear conditioning paradigm at which animals were sacrificed and immunostained for c-Fos. (B, D) Representative photomicrographs obtained with confocal microscopy 10 µm z-stack projections of the ventral hippocampus CA1 pyramidal layer and basolateral amygdala. (C, E) Quantification of Thy1-YFP/c-FOS double positive neurons in the Thy1-YFP/*NTRK3*-FC and Thy1-YFP/WT-FC animals (n = 5) in the ventral hippocampus CA1 and CA3 pyramidal layers and basolateral

amygdala. Student's t-test was used for comparisons and statistical significance was set a * $p < 0.05$. Thy1, thymus cell antigen 1; YFP, yellow fluorescent protein; WT, wild type; Tg*NTRK3*, transgenic mice overexpressing *NTRK3*; FC, fear conditioned; CA1py-CA3py, *Cornu Ammonis pyramidal layers*; BLA, basolateral amygdala.

4.2.4 Activation pattern of hippocampal, amygdala and medial prefrontal cortex excitatory neurons, upon extinction of contextual fear in Tg*NTRK3*

To characterize the specific activation pattern of excitatory neurons in the process of extinction of contextual fear memory we quantified the number of Thy1-YFP/c-FOS double positive neurons (activated excitatory neurons) in the mPFC (Figure 11B), hippocampus (Figure 11D) and amygdala (Figure 11F) of Thy1-YFP/*NTRK3* and Thy1-YFP/WT mice, sacrificed one hour after contextual fear extinction (Figure 11A). Thy1-YFP/*NTRK3*-Ext mice showed a lower number of Thy1-YFP/c-FOS double-positive cells, as compared to Thy1-YFP/WT-Ext in the IL region of the mPFC (Figure 11C, Student's t-test p = 2.3E-04) with no differences found in the PL (Figure 11C, p = 0.32). In contrast, in the ventral hippocampus CA1py layer the number of Thy1-YFP/c-FOS double-positive cells was higher in Thy1-YFP/*NTRK3*-Ext than in their respective WT littermates (Figure 11E, Student's t-test p = 4.6E-04), with no differences found between genotypes in CA3py layer (Figure 11E, p = 0.08) and no differences in both CA1py and CA3py layers of the dorsal hippocampus (data not shown). In the BLA nuclei of amygdala no differences were found between genotypes in the number of activated excitatory neurons. (Figure 11G, p = 0.7).

The increased CA1py activation and the lack of activation in the IL described in the previous c-FOS experiments match with excitatory cells, in both brain areas of Tg*NTRK3* mice.

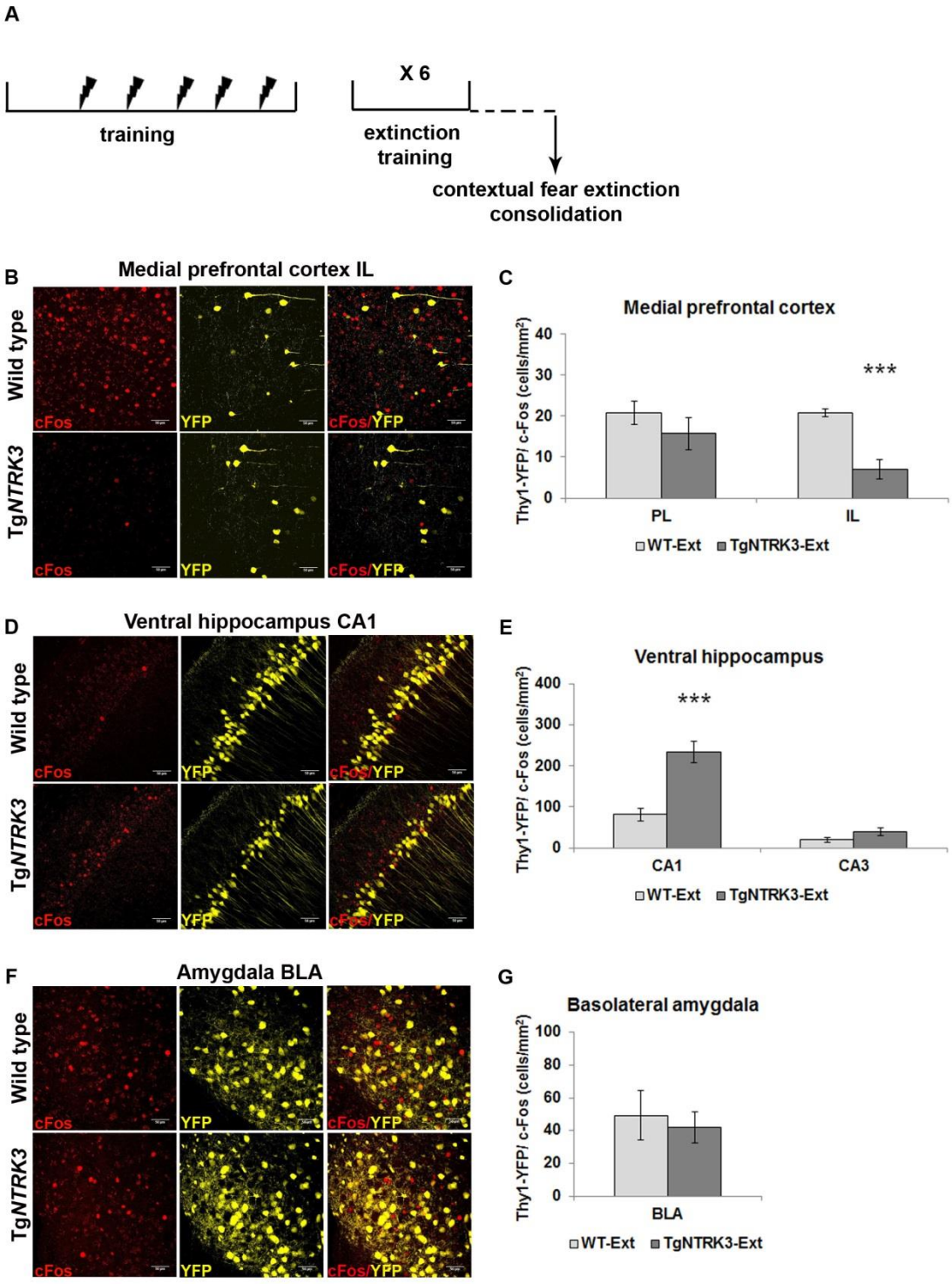

Figure 11: Activation pattern of excitatory neurons in the medial prefrontal cortex, hippocampus and amygdala of Thy1-YFP/WT and Thy1-YFP/*NTRK3* animals after extinction of contextual fear. (A) Schematic representation of the time point during the pure contextual fear extinction paradigm at which animals were sacrificed and immunostained for c-

FOS. (B, D, F) Representative photomicrographs obtained with confocal 10 µm z-stack projections of the medial prefrontal cortex IL, ventral hippocampus CA1py and basolateral amygdala. (C, E, G) Quantification of Thy1-YFP/c-FOS double positive neurons in the Thy1-YFP/*NTRK3*-Ext (n =5) and Thy1-YFP/WT-Ext animals (n = 6) in the medial prefrontal cortex PL and IL, ventral hippocampus CA1py and CA3py layers and basolateral amygdala. Student's t-test was used for comparisons and statistical significance was set a $p < 0.05$, *** $p < 0.001$. Thy1, thymus cell antigen 1; YFP, yellow fluorescent protein; WT, wild type; Tg*NTRK3*, transgenic mice overexpressing *NTRK3*; Ext, fear extinction; PL, pre-limbic; IL, infra-limbic; CA1py-CA3py, *Cornu Ammonis pyramidal layers*; BLA, basolateral amygdala.

4.3 Role of the glutamatergic and GABAergic systems in the Tg*NTRK3* mice

The altered activation pattern found in the hippocampus – amygdala – prefrontal cortex of Tg*NTRK3* mice during fear learning suggests a potential involvement of the excitatory and inhibitory system. We proceed with the identification of putative cellular and molecular players, focusing on glutamatergic and GABAergic systems.

4.3.1 Analysis of the glutamatergic population in the hippocampus, amygdala and prefrontal cortex brain regions of Tg*NTRK3* mice

Using the double transgenic mice Thy1-YFP/*NTRK3* and Thy1-YFP/WT littermates we quantified the number of glutamatergic cells per area in each genotype. Student's t-test statistical analysis in each region revealed no differences in the number of Thy1-YFP positive cells per area between genotypes in the mPFC (Figure 12A, B, PL $p = 0.055$; IL $p = 0.19$), ventral hippocampus (Figure 12C, D, DG $p = 0.59$; CA1 $p = 0.36$; CA3 $p = 0.11$) or amygdala (Figure 12E, F, $p = 0.13$) brain regions.

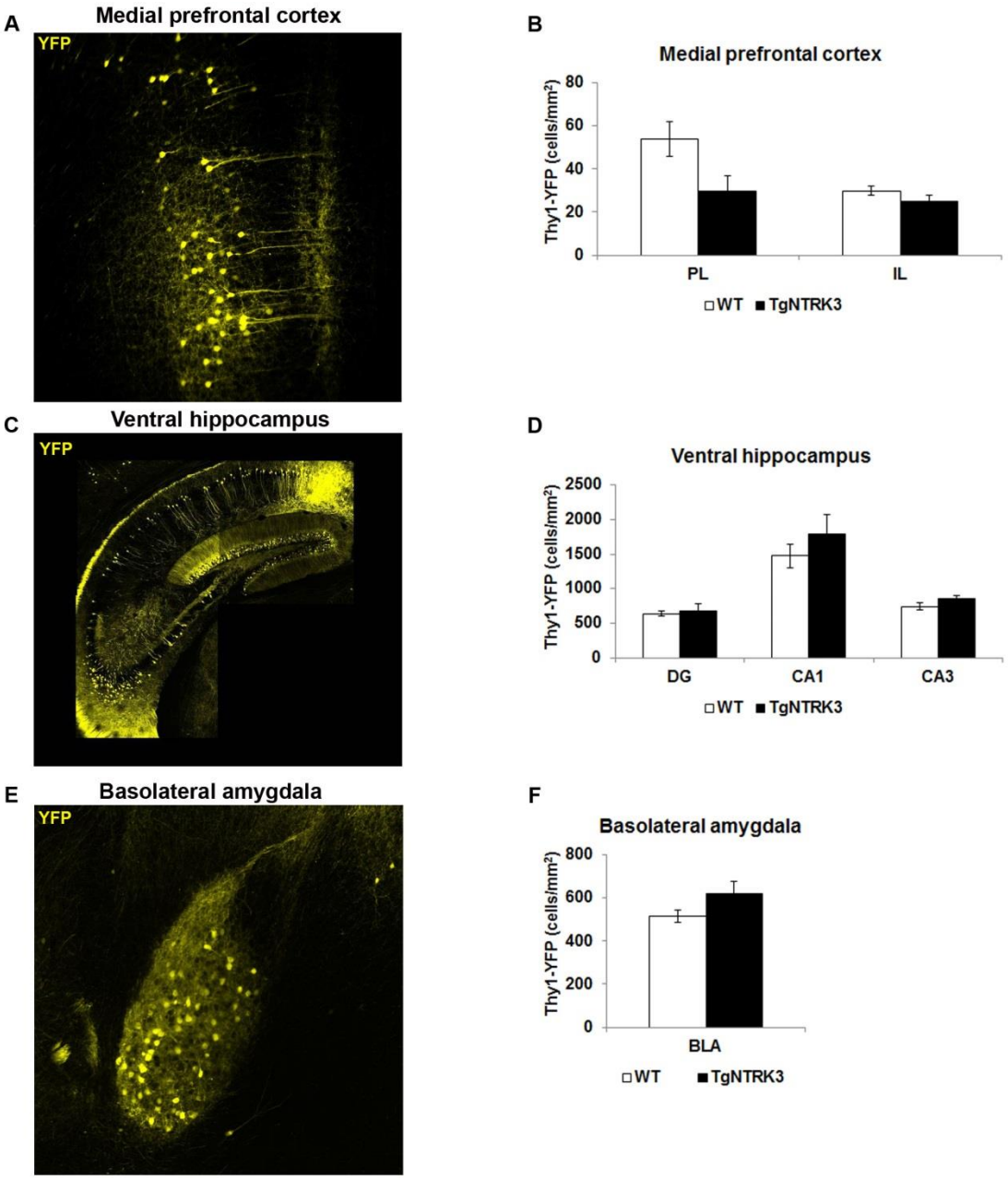

Figure 12: Thy1-YFP/WT and Thy1-YFP/*NTRK3* glutamatergic neuronal densities in the medial prefrontal cortex, hippocampus and amygdala brain areas. Representative photomicrographs obtained with confocal 10 µm z-stack projections of the (A) medial prefrontal cortex, (C) ventral hippocampus and (E) basolateral amygdala. Quantification of Thy1-YFP positive neurons per area in the Thy1-YFP/WT (n = 6) and Thy1-YFP/*NTRK3* (n = 5) animals in the (B) medial prefrontal cortex, (D) ventral hippocampus and (F) basolateral amygdala. Statistical analysis was performed with Student's t-test for each region analyzed and statistical significance was set at $p < 0.05$. Thy1, thymus cell antigen 1; YFP, yellow fluorescent protein; WT, wild type; Tg*NTRK3*, transgenic mice overexpressing *NTRK3*; PL, pre-limbic; IL, infra-limbic; DG, dentate gyrus; CA1-CA3, *Cornu Ammonis*; BLA, basolateral amygdala.

4.3.2 GABAergic cell population in the hippocampus, amygdala and prefrontal cortex brain regions of Tg*NTRK3* mice

The total number of GABAergic neurons was quantified by analyzing WT and Tg*NTRK3* brain slices immunostained against the rate-limiting enzyme in the synthesis of GABA (GAD65/67). Tg*NTRK3* brains did not show any statistically significant difference in the density of GAD65/67 positive neurons / area, as compared to WT, in any layer or sub-region analyzed in the mPFC (Figure 13A, B), hippocampus (Figure 13C, D) or amygdala (Figure 13E, F) (Student's t-test).

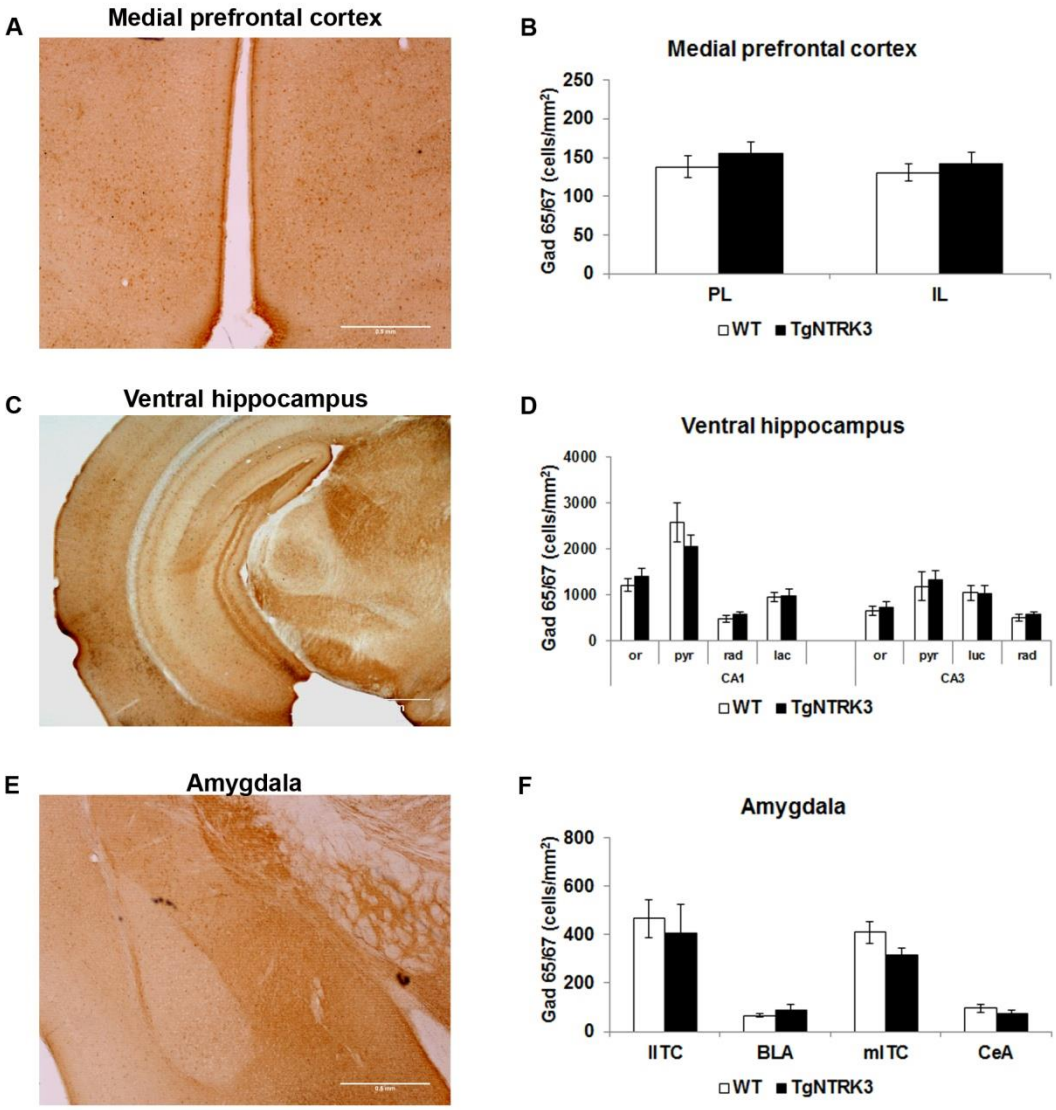

Figure 13: WT and Tg*NTRK3* GABAergic neuronal density in the medial prefrontal cortex, the amygdala and the hippocampus in naïve conditions. Representative photomicrographs of the (A) medial prefrontal cortex, (C) ventral hippocampus and (E) amygdala immunostained for GAD65/67. Quantification of GAD65/67 positive neurons in WT and Tg*NTRK3* animals (n = 5 per genotype) in the (B) medial prefrontal cortex, (D) ventral hippocampus and (F) amygdala. Student's t-test was used for comparisons and statistical significance was set at $p < 0.05$. GAD 65/67, glutamic acid decarboxylase isoforms 65 kD and 67 kD; WT, wild type; Tg*NTRK3*, transgenic mice overexpressing *NTRK3*; PL, pre-limbic; IL, infra-limbic; Or, *stratum oriens*; py, *stratum pyramidale*; rad, *stratum radiatum*; CA1-CA3, *Cornu Ammonis*; lITC, lateral intercalated cell cluster; BLA, basolateral amygdala; mITC, medial intercalated cell cluster; CeA, central amygdala.

4.3.3 Excitatory and inhibitory presynaptic balance in the hippocampus of Tg*NTRK3*

The hyperactivation observed in the hippocampus of Tg*NTRK3* mice that corresponds with a higher number of activated excitatory neurons suggests a disruption of the excitation / inhibition balance. A possible explanation of this could be found in altered synaptic inputs arriving to pyramidal neurons.

Using double immunofluorescent staining for the presynaptic VGLUT1 and VGAT markers we quantified the number of puncta in CA1 and CA3 hippocampal pyramidal apical dendrites. Tg*NTRK3* mice showed a significant increase of excitatory presynaptic terminals, as shown by the increased VGLUT1/VGAT ratio in CA1rad (Student's t-test $p = 0.019$), but not in CA3rad ($p = 0.45$) of ventral hippocampus (Figure 14A, B).

Next, we quantified the number of excitatory synapses using double immunofluorescence for presynaptic VGLUT1 and postsynaptic PSD95 excitatory markers. Tg*NTRK3* mice did not show a statistically significant difference in the VGLUT1/PSD95 co-localization, as compared to WT littermates (Figure 14C, D).

Overall, these results suggest an unbalanced excitation to inhibition ratio in CA1 pyramidal neurons of Tg*NTRK3* towards an increased excitatory synaptic load. Within a context of normal excitatory synaptic contacts, the Tg*NTRK3* CA1py neurons might be hyperactivated because of an increased presynaptic glutamate vesicle fill and, probably, release. This confirms the hyperexcitability

in the CA3-CA1 hippocampal sub-circuit and is in line with the enhanced and sustained long-term potentiation previously observed in Tg*NTRK3* mice.

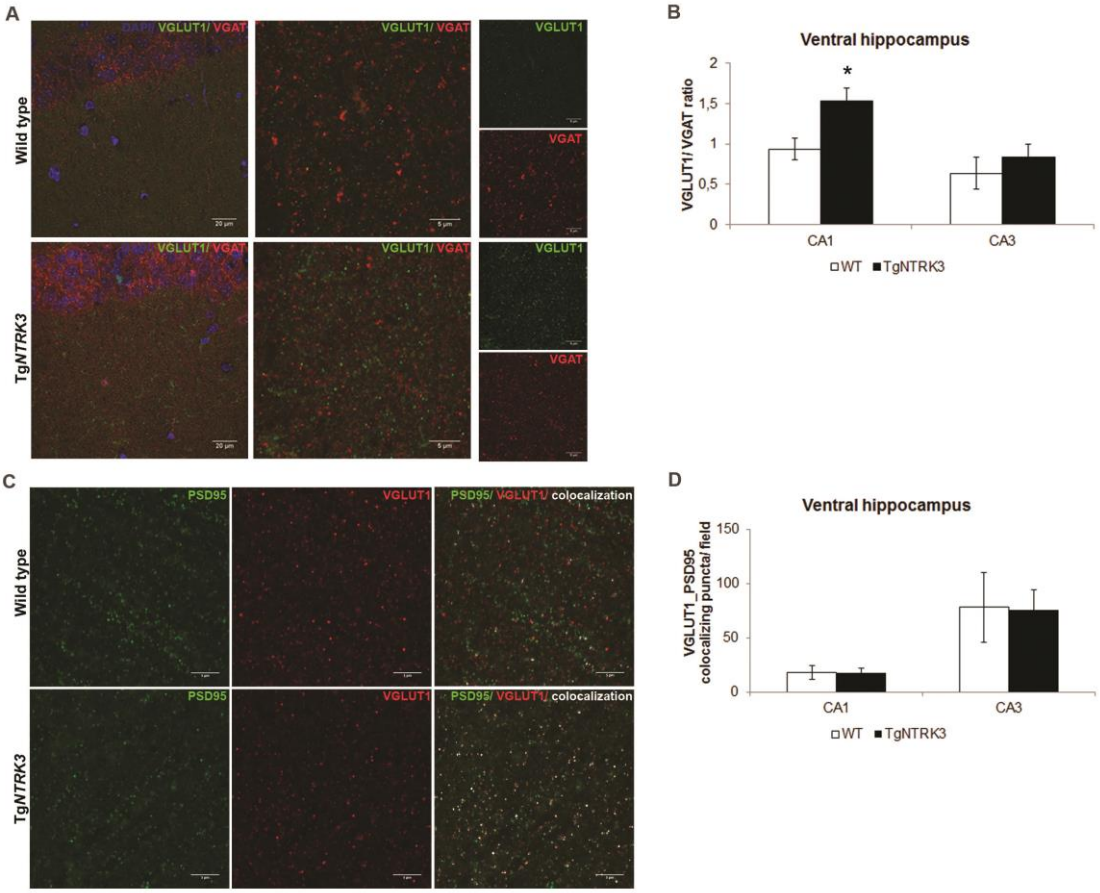

Figure 14: Excitatory vs. inhibitory synaptic balance and excitatory synaptic contacts in the hippocampus of WT and Tg*NTRK3* naïve mice. (A) Representative photomicrographs of CA1 *stratum radiatum* stained for VGLUT1 and VGAT and (B) quantification of VGLUT1/VGAT puncta ratio in the CA1 and CA3 *stratum radiatum* in WT (n = 7) and Tg*NTRK3* (n = 9) mice. (C) Representative photomicrographs of CA1 *stratum radiatum* staining for VGLUT1 and PSD95 and (B) quantitative analysis of VGLUT1/PSD95 co-localizing puncta in the CA1 and CA3 *stratum radiatum* in WT and Tg*NTRK3* mice (n = 8 per genotype). Student's t-test was used for comparison and statistical significance was set at * $p < 0.05$. VGLUT1, vesicular glutamate transporter type 1; VGAT, vesicular GABA transporter; PSD95, post-synaptic density protein 95; WT, wild type; Tg*NTRK3*, transgenic mice overexpressing *NTRK3*; CA1-CA3, *Cornu Ammonis*.

4.3.4 Pharmacological rescue of enhanced contextual fear memory in Tg*NTRK3* mice: tackling the glutamatergic system

Pharmacological intervention can re-balance the excitatory/inhibitory system thus putatively rescuing the enhanced contextual fear memory in Tg*NTRK3* mice. Here we performed a series of experiments in which we blocked the glutamatergic system.

A dose-response curve was generated upon i.p. administration of ifenprodil (a selective antagonist of the NR2B subunit of NMDA receptors), at different doses (0.5, 1, 5 and 10 mg/Kg) in WT animals immediately after pure contextual fear conditioning training. Contextual fear memory was measured 24-hours later (Figure 15A). All groups presented the same levels of freezing in the acquisition session, before the drug administration (Figure 15B, repeated measures 1-way ANOVA, session effect, p = 8.6E-19, treatment effect p = 0.73). No differences were found in the freezing response upon administration of different doses of ifenprodil during the test or re-test sessions (Figure 15C, 1-way ANOVA, treatment effect, test, p = 0.2, re-test, p = 0.2). However, although not statistically significant, a reduction in freezing time was observed in the test session in the group treated with ifenprodil 1 mg/Kg.

Based on the preliminary results obtained in the doses-curve we selected the dose of ifenprodil 1 mg/Kg to perform the experiment itself. Both WT and Tg*NTRK3* mice performed equally during the acquisition phase of the contextual fear-conditioning paradigm, presenting equivalent levels of freezing (Figure 15D, repeated measures 2-way ANOVA, session effect, p = 4.6E-10, genotype effect, p = 0.91, treatment effect, p = 0.82). Post training administration of ifenprodil did not reduce fear memory in any genotype during the test and re-test sessions (Figure 15E, 2-way ANOVA, treatment effect test, p = 0.38, re-test p = 0.25). Moreover, Tg*NTRK3* mice show increased freezing levels during the test session, both saline and 1 mg/Kg ifenprodil groups (Figure 15E, 2-way ANOVA, genotype effect p = 0.023) not further observed during the re-test session (Figure 15E, 2-way ANOVA, genotype effect p = 0.98). This experiment suggests that the enhanced contextual fear memory in Tg*NTRK3*

mice cannot be rescued by antagonizing the NR2B receptor, at least not with the dose of ifenprodil used here.

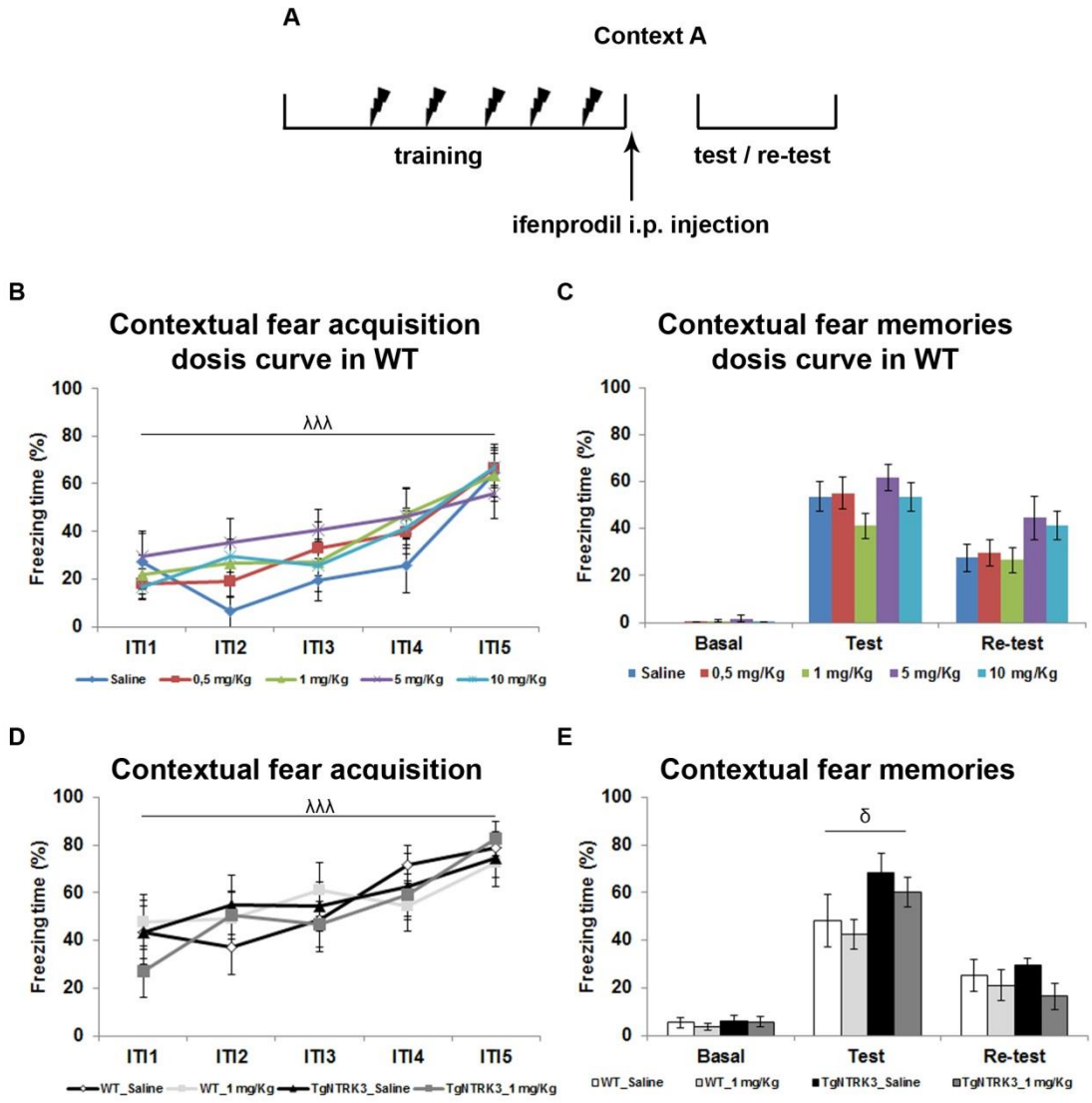

Figure 15: Effect of ifenprodil on contextual fear memory of WT and Tg*NTRK3* mice. (A) Schematic representation of the pure contextual fear conditioning paradigm and systemic administration of ifenprodil after the training session. (B, C) Dose-response curve. Freezing response of WT animals treated with saline (n = 8) or ifenprodil at 0.5 mg/Kg (n = 11), 1 mg/Kg (n = 12), 5 mg/Kg (n =10) and 10 mg/Kg (n = 11) during the (B) training session and (C) test and re-test sessions. (D, E) Effect of ifenprodil on contextual fear memory. Percentage of freezing of WT-saline (n = 9), WT-ifenprodil 1mg/Kg (n = 10), Tg*NTRK3*-saline (n = 10) and Tg*NTRK3*-ifenprodil 1mg/Kg (n = 11) in the (D) training session and (E) test and re-test sessions. We used repeated measures 1-way ANOVA (training session) and 1-way ANOVA (test and re-test sessions) for the dose-curve and for the experiment itself we used repeated measures 2-way

ANOVA with sessions as within subject factor and genotype and treatment as between subject factors (training session) and 2-way ANOVA with genotype and treatment as factors (test and re-test sessions). $^{\delta}$ genotype effect $p < 0.05$, $^{\lambda\lambda\lambda}$ session effect $p < 0.001$. WT, wild type; Tg*NTRK3*, transgenic mice overexpressing *NTRK3*.

4.3.5 Pharmacological rescue of enhanced contextual fear memory in Tg*NTRK3* mice: tackling the GABAergic system

Next, we attempted to re-balance the excitation/inhibition equilibrium by potentiating the action of GABA at the synapse, thereby counteracting the excitatory overload. Tiagabine is a GABA reuptake inhibitor, acting at the presynaptic GABA transporter (GAT). We first generated a dose-response curve by injecting WT animals with tiagabine (i.p.) at different doses (2.5, 5 and 10 mg/Kg) immediately after pure contextual fear conditioning training and measuring contextual fear memory 24-hours later (Figure 16A).

All groups presented a similar performance in the acquisition session of the fear-conditioning paradigm, before drug administration (Figure 16B, repeated measures 1-way ANOVA, session effect, p = 3.4E-08, treatment effect p = 0.42). Also, no significant differences in the freezing levels were observed among groups receiving either saline or the different doses of tiagabine at the test and re-test sessions (Figure 16C, 1-way ANOVA, treatment effect, test, p = 0.24, re-test, p = 0.77). However, although not statistically significant, the most important reduction of freezing response was observed in the group treated with tiagabine 10 mg/Kg. Therefore we selected this dose for experiment in Tg*NTRK3*.

Both WT and Tg*NTRK3* mice equally acquired a freezing response in the training session of the contextual fear-conditioning paradigm (Figure 16D, repeated measures 2-way ANOVA, session effect, p = 3.6E-10, genotype effect, p = 0.68, treatment effect, p = 0.7). Interestingly, tiagabine administration immediately after the training session reduced the freezing response specifically in Tg*NTRK3* mice, to levels comparable to WT (Figure 16E, 2-way ANOVA, genotype x treatment interaction p = 0.02; *post hoc* Bonferroni Tg*NTRK3*-saline vs. Tg*NTRK3*-tiagabine p = 6.9E-06, WT-tiagabine vs. Tg*NTRK3*-tiagabine p = 0.036, WT-saline vs. Tg*NTRK3*-saline p = 0.010). A similar trend, although not

reaching statistical significance, was observed during the re-test session (Figure 16E, genotype x treatment interaction p = 0.16).

To conclude, the enhanced contextual fear memory detected in Tg*NTRK3* can be rescued by enhancing GABAergic transmission, through the blockade of GABA reuptake at the synapse.

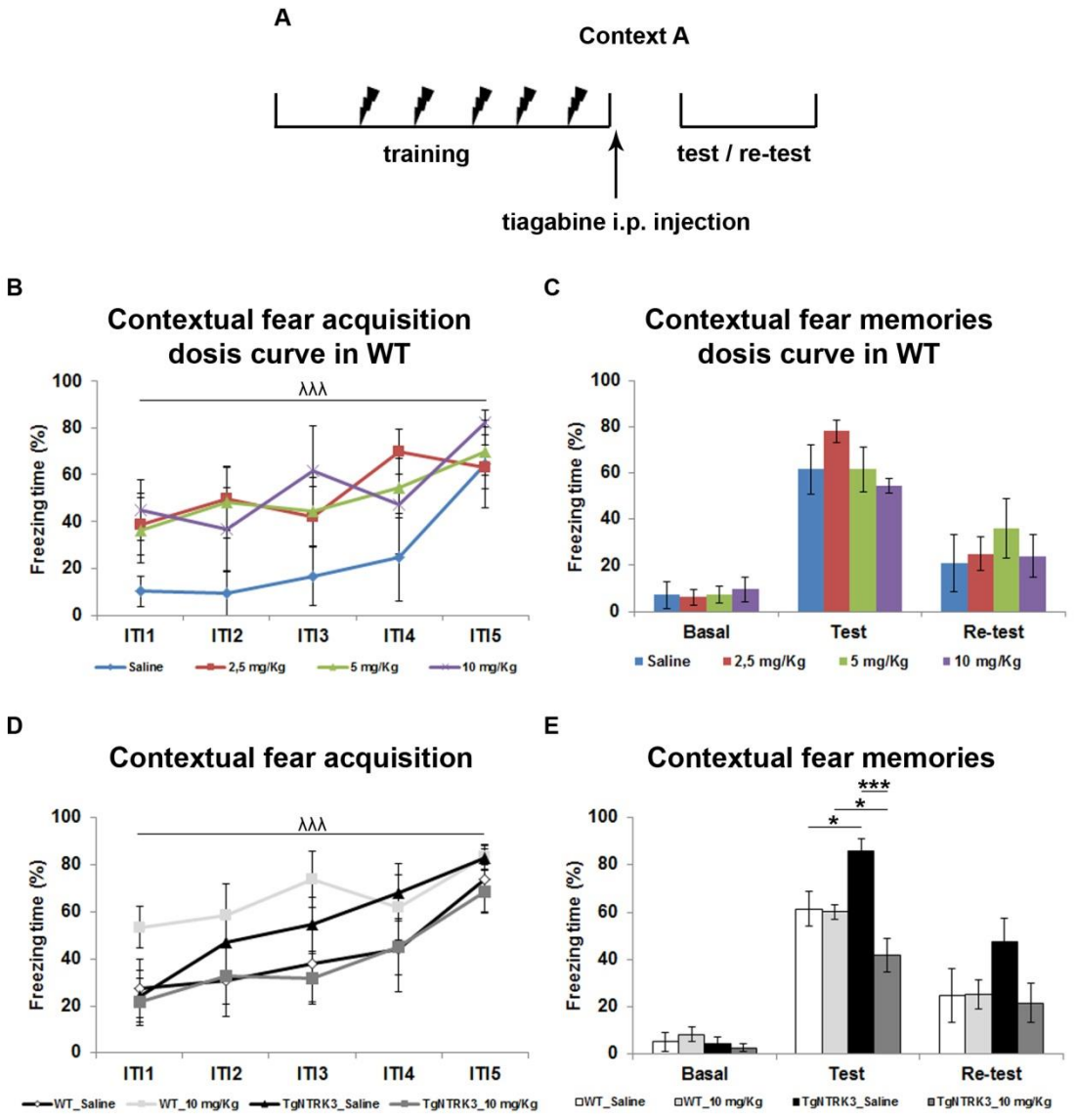

Figure 16: Effect of tiagabine on contextual fear memory of WT and Tg*NTRK3* mice. (A) Schematic representation of the pure contextual fear conditioning paradigm and systemic administration of tiagabine after the training session. (B, C) Dose-response curve. Freezing response of WT animals treated with saline (n = 5) or tiagabine at 2.5 mg/Kg (n = 7), 5 mg/Kg (n = 8) and 10 mg/Kg (n =5) doses during the (B) training session and (C) test and re-test sessions. (D, E) Effect of tiagabine on contextual fear memory. Percentage of freezing of WT-

saline (n = 7), WT-tiagabine 10mg/Kg (n = 8), Tg*NTRK3*-saline (n = 9) and Tg*NTRK3*-tiagabine 10mg/Kg (n =10) in the (D) training session and (E) test and re-test sessions. We used repeated measures 1-way ANOVA (training session) and 1-way ANOVA (test and re-test sessions) for the dose-curve and for the experiment itself we used repeated measures 2-way ANOVA with sessions as within subject factor and genotype and treatment as between subject factors (training session) and 2-way ANOVA with genotype and treatment as factors (test and re-test sessions), *post hoc* Bonferroni upon genotype x treatment interaction * $p < 0.05$, *** $p < 0.001$. $^{\delta}$ genotype effect $p < 0.05$, $^{\lambda\lambda\lambda}$ session effect $p < 0.001$. WT, wild type; Tg*NTRK3*, transgenic mice overexpressing *NTRK3*.

4.3.6 Pharmacological proof of hippocampal-dependence of the enhanced contextual fear memory in Tg*NTRK3* mice

In order to evaluate whether Tg*NTRK3* enhanced fear memory was due to an excitatory/inhibitory dysbalance in the ventral hippocampus, mice were infused with tiagabine, through locally implanted injection cannulae, after the training session of contextual fear-conditioning.

To confirm injection sites, brains were sliced, counterstained with haematoxilyn and injection sites confirmed under the microscope (Figure 17B).

In the training session, no significant differences between groups were observed in freezing levels (Figure 17C, repeated measures 2-way ANOVA, session effect, $p = 0.02$, genotype effect, $p = 0.86$, treatment effect, $p = 0.65$). Tiagabine infusion reduced the freezing response in both WT and Tg*NTRK3* mice during the test session (Figure 17D, 2-way ANOVA, treatment effect $p = 0.005$). During the re-test, WT animals treated with saline reduced their freezing response and tiagabine infusion did not change their freezing levels. However while saline treated Tg*NTRK3* mice showed enhanced freezing behaviour (as previously observed, see section 4.1.1, 4.3.4 and 4.3.5) this enhanced fear was completely rescued by tiagabine (Figure 17D, 2-way ANOVA, genotype x treatment interaction $p = 0.06$, *post hoc* Bonferroni, WT-saline vs. Tg*NTRK3*-saline $p = 0.014$, Tg*NTRK3*-saline vs. Tg*NTRK3*-tiagabine $p = 0.0026$).

Increasing the GABAergic load locally in the ventral hippocampus is thus sufficient to rescue Tg*NTRK3* enhanced contextual fear memory at long term.

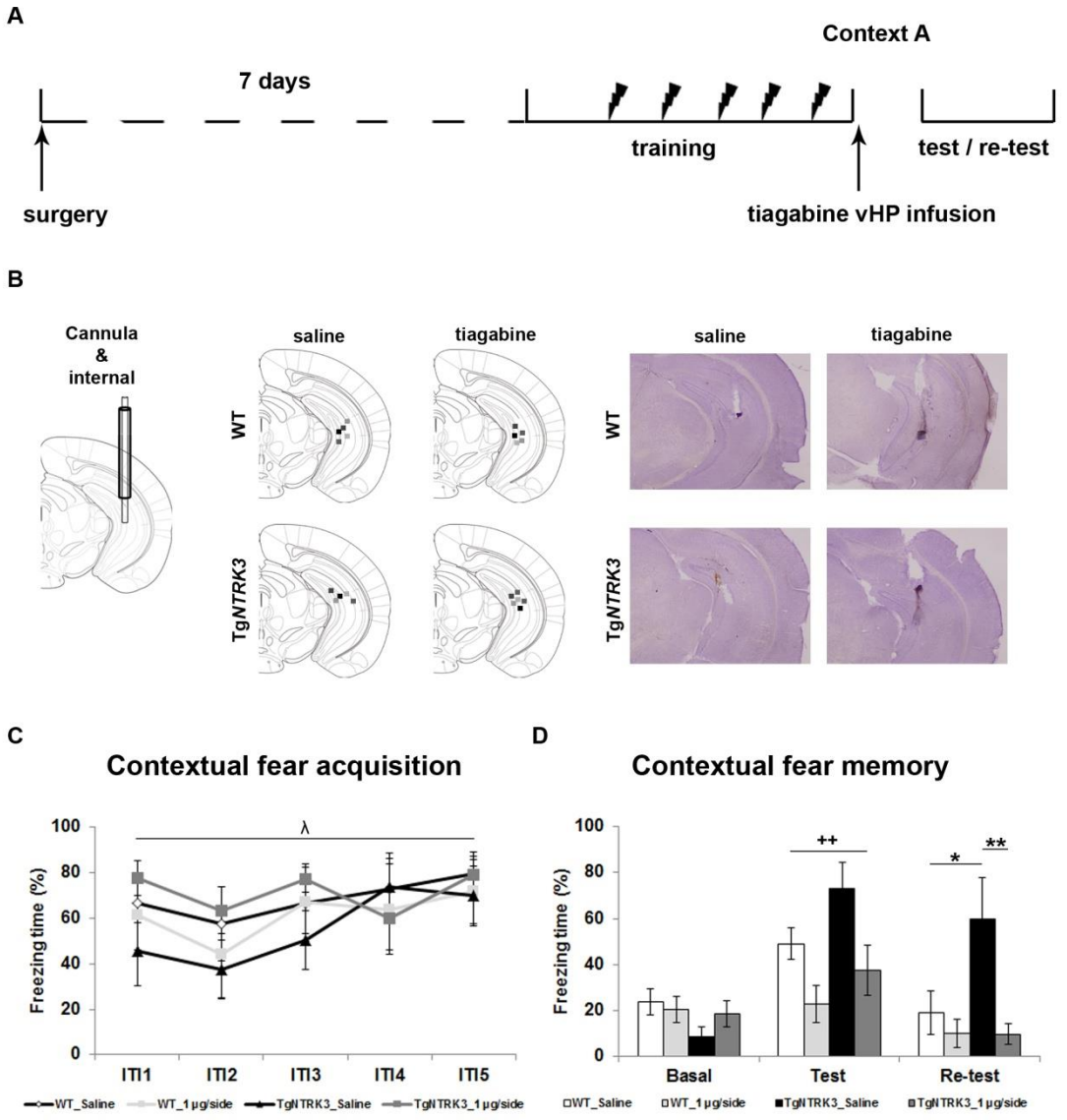

Figure 17: Effect of intra-ventral hippocampus tiagabine administration on contextual fear memory of WT and Tg*NTRK3* mice. (A) Schematic representation of the stereotaxic surgical cannulae implantation and pure contextual fear conditioning paradigm with intra-ventral hippocampus infusion of tiagabine after the training session. (B) Schematic representation of the brain region in which cannula was implanted and infusion was performed (stereotaxic coordinates: AP = -3.00, ML = ±3.00, DV = -2.3), real injection sites for each animal used in the experiment infused with either saline or tiagabine (WT-saline n = 5, WT- tiagabine 1µg/side n = 5, Tg*NTRK3*-saline n = 5 and Tg*NTRK3*-tiagabine 1µg/side n = 6) and representative histological sections. (C-D) Effect of intra-vHP tiagabine infusion on contextual fear memory. Percentage of freezing time spent by WT and Tg*NTRK3* mice treated with either saline or tiagabine during the (C) training session and (D) test and re-test sessions. We used repeated measures 2-way ANOVA with sessions as within subject factor and genotype and treatment as between subject factors (training session) and 2-way ANOVA with genotype and treatment as

factors (test and re-test sessions), *post hoc* Bonferroni upon genotype x treatment interaction * $p < 0.05$, ** $p < 0.01$, $^{\lambda}$ session effect $p < 0.05$; $^{++}$ treatment effect $p < 0.01$. vHP, ventral hippocampus; ITI, inter trial interval; WT, wild type; Tg*NTRK3*, transgenic mice overexpressing *NTRK3*.

4.4 NT3 and TrkC during contextual fear processes in Tg*NTRK3* mice

Fear memories are dynamic learning processes, requiring activity dependent plasticity mechanisms in the hippocampus - amygdala - medial prefrontal cortex circuit. Several evidences highlighted the importance of neurotrophins and their receptors in fear memories, such as BDNF in the HP – mPFC circuit as a plasticity mediator inducing extinction of fear memories, but much less is known about the role of NT3.

In this section we characterized putative adaptive changes in NT3 and its receptor, TrkC, along contextual fear memory processes. In Tg*NTRK3* mice, the genetic overexpression of TrkC, might finally cause differential alterations in its function and in the function of its ligand NT3 during fear. We characterized the NT3-TrkC neurotrophin system by measuring its expression levels in naïve WT and Tg*NTRK3* mice and upon fear conditioning and fear extinction training.

4.4.1 Quantification of TrkC and NT3 protein levels during contextual fear memories

We quantified the protein expression levels of TrkC, by western blot and NT3, by ELISA from protein extracts of hippocampus and prefrontal cortex of WT and Tg*NTRK3* mice in naïve, contextual FC and FExt states (Fig. 18A).

4.4.1.1 TrkC

In the hippocampus WT and Tg*NTRK3* mice in naïve condition showed no differences in the expression level of the full length TrkC isoform, whereas fear conditioning increased significantly the expression of TrkC in both WT and Tg*NTRK3* mice, as compared to naïve condition (Figure 18B, D, 2-way ANOVA, FC effect $p = 0.001$). Similarly, in the prefrontal cortex, Tg*NTRK3* mice in naïve

condition showed similar expression levels of TrkC, as compared to naïve WT littermates and fear conditioning increased its expression in both genotypes (Figure 18C, E, 2-way ANOVA, FC effect p = 0.0025). In addition TrkC levels were higher in TgNTRK3 mice (Figure 18C, E, 2-way ANOVA, genotype effect p = 0.029), mostly due to higher expression upon FC.

Fear extinction training induced in the hippocampus a significant increase in the expression of full length TrkC isoform in WT mice as compared to naïve condition but not in Tg*NTRK3* (Figure 18B, D, 2-way ANOVA, genotype x FExt interaction effect p = 0.04; Bonferroni *post hoc* test, WT-Naive vs. WT-FExt p = 0.018, WT-FExt vs. Tg*NTRK3*-FExt p = 0.0016). In the prefrontal cortex fear extinction induced increase in the TrkC expression in both genotypes (Figure 18C, E, 2-way ANOVA, FExt effect p = 0.006).

To sum up, FC increases TrkC expression levels similarly in WT and Tg*NTRK3* mice in both the hippocampus and the mPFC. FExt increases TrkC level in the hippocampus of WT animals, but not in Tg*NTRK3* littermates, while similar increases were observed in the mPFC of both genotypes. Overall the TrkC protein seem to be directly involved in the HP and mPFC during contextual fear learning processes, and a reduction of TrkC within the HP might contribute to the impaired extinction phenotype of Tg*NTRK3* mice.

4.4.1.2 NT3

The quantitative analysis of NT3 showed that the hippocampus of WT and Tg*NTRK3* mice present equal amounts of protein during naïve condition and fear conditioning had no effect on these levels (Figure 18F). However the same FC process had opposite effects in the prefrontal cortex of the two genotypes, resulting in an increase in WT and a decrease in Tg*NTRK3* (Figure 18G, 2-way ANOVA, genotype x FC interaction, p = 0.038 with no further significative Bonferroni *post hoc* test). Although statistically supported by a tendency, it appears that NT3 levels are higher in Tg*NTRK3* mice in naïve condition as compared to naïve WT littermates (WT-Naïve vs. Tg*NTRK3*-Naïve, p = 0.16) and FC increased NT3 levels in WT (WT-Naïve vs. WT-FC, p = 0.10) but

reduced it in Tg*NTRK3* (Tg*NTRK3*-Naïve vs. Tg*NTRK3*-FC, p = 0.16), as compared to naïve condition.

Fear extinction did not change the levels of NT3 in the hippocampus of neither WT nor Tg*NTRK3* mice (Figure 18F). In the mPFC FExt induced a tendency to differentially affect NT3 amounts in the two genotypes, resulting in a reduction in Tg*NTRK3* mice, but not in WT littermates (Figure 18G, 2-way ANOVA, genotype x FExt interaction, p = 0.1 Bonferroni *post hoc* test: WT-Naive vs. WT-FExt, p = 0.8, Tg*NTRK3*-Naive vs. Tg*NTRK3*-FExt, p = 0.024).

To sum up, these results indicate that Tg*NTRK3* mice experience a lack of NT3 amount during contextual fear memory formation and, more importantly, extinction in their prefrontal cortical area.

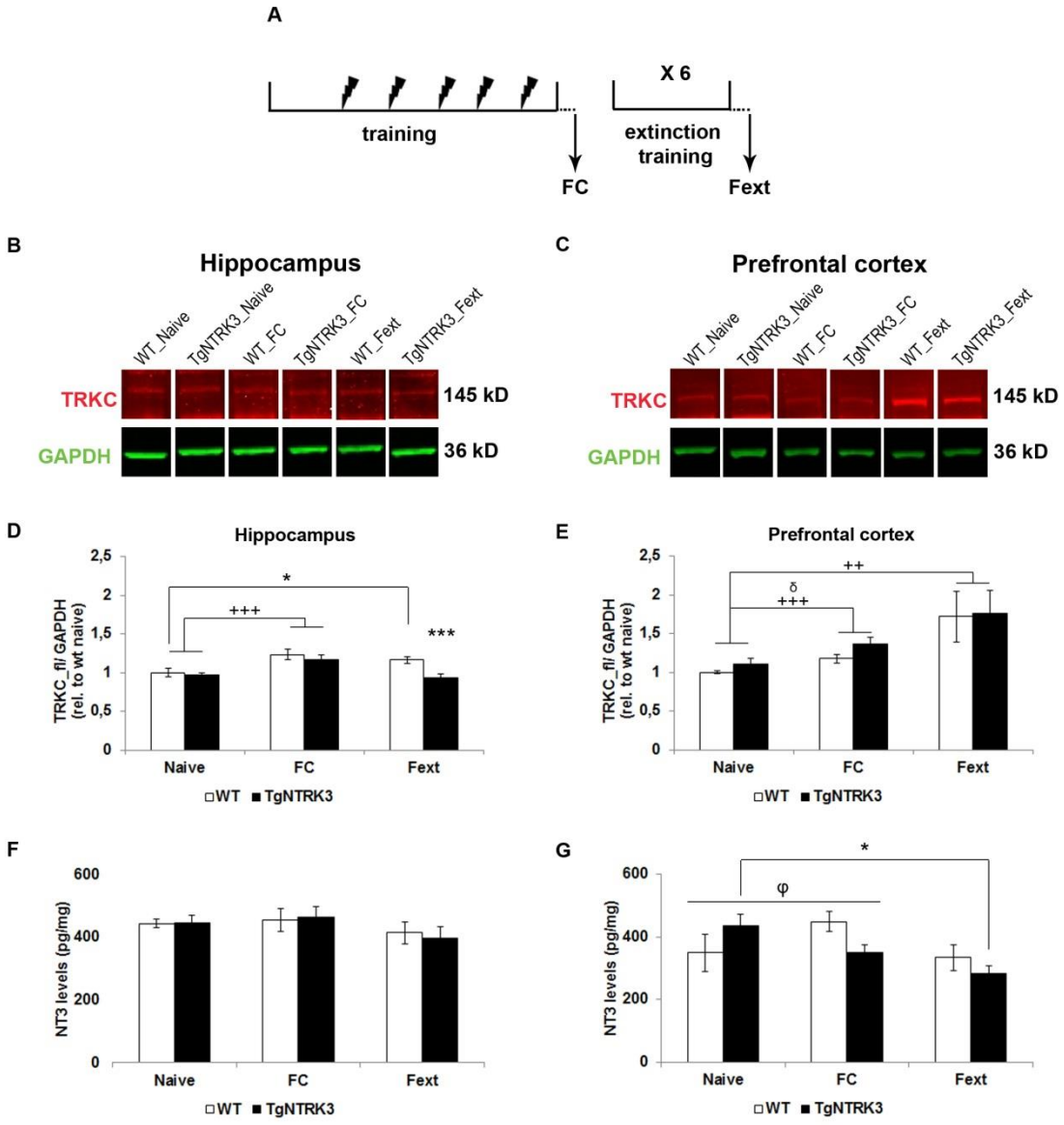

Figure 18: WT and Tg*NTRK3* expression levels of TrkC and NT3 in the hippocampus and medial prefrontal cortex in naive condition, upon fear conditioning and fear extinction. (A) Schematic representation of the pure contextual fear conditioning and extinction time points at which animals were sacrificed and protein extracted from hippocampus and mPFC for expression analysis of TrkC and NT3 protein levels. (B, C) Representative western blot bands of TrkC full length isoform and GAPDH housekeeping proteins from protein extracts of (B) hippocampus and (C) prefrontal cortex of WT and Tg*NTRK3* mice sacrificed in naïve, FC and FExt states. (D, E) Quantitative analysis of the TrkC_fl/GAPDH protein ratio in (D) hippocampus and (E) prefrontal cortex of WT-Naive, Tg*NTRK3*-Naive, WT-FC, Tg*NTRK3*-FC, WT-FExt and Tg*NTRK3*-FExt mice (n = 6 per group). (F, G) Quantitative analysis of the NT3 protein levels, by ELISA in (F) hippocampus and (G) prefrontal cortex of WT-Naive, Tg*NTRK3*-Naive, WT-FC, Tg*NTRK3*-FC, WT-FExt and Tg*NTRK3*-FExt mice (n = 5 per group). We used 2-way ANOVA

with genotype and state (naïve vs. FC and naïve vs. Fext) for each brain region, *post hoc* Bonferroni upon genotype x state interaction * $p < 0.05$, *** $p < 0.001$; $^{++}$ treatment effect $p < 0.01$, $^{+++}$ treatment effect $p < 0.001$; $^{\delta}$ genotype effect $p < 0.05$; $^{\varphi}$ genotype x state interaction $p < 0.05$. vHP, ventral hippocampus. TrkC_fl, tyrosine kinase receptor type 3 full length isoform; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; NT3, neurotrophin type 3; WT, wild type; Tg*NTRK3*, transgenic mice overexpressing *NTRK3*; FC, fear condition; Fext, fear extinction.

4.4.2 Rescue of the impaired contextual fear extinction memory in Tg*NTRK3* mice: intra-mPFC NT3 facilitation of extinction

The impaired extinction of contextual fear memory in Tg*NTRK3* mice is associated with a reduced expression of NT3 in the mPFC, a region showing increased levels of its receptor, TrkC. This could probably result in a lack of NT3-TrkC activation pathway, an impaired mechanism inducing plasticity in the mPFC, such as MAPK/ERK-mediated extinction learning (see introduction section 1.7). In order to evaluate whether Tg*NTRK3* impaired fear extinction memory was directly caused by this mechanism, mice were infused with 0.75 µg of NT3 per side in the mPFC, through locally implanted injection cannulae, after the extinction training session of the pure contextual fear extinction paradigm (Figure 19A).

In brains slices, counterstained with haematoxilyn, microscopic analysis of injection sites (Figure 19B), confirmed that were for all groups mostly localized within the correct medial portion of the prefrontal cortex. During the extinction training session, a significant difference between genotypes was observed in freezing levels, with WT groups reducing freezing progressively along trials and Tg*NTRK3* littermates maintaining high levels of freezing throughout the entire session (Figure 19C, repeated measures 2-way ANOVA, genotype effect, $p = 1.6E-04$). Post-training NT3 infusion had opposite effects on extinction memory test in the two genotypes: it increased the freezing response in WT animals as compared to WT infused with saline and, importantly, NT3 in Tg*NTRK3* mice reduced freezing to very low levels, rescuing their impaired extinction memory (Figure 19D, 2-way ANOVA, genotype x treatment interaction $p = 3E-04$, *post hoc* Bonferroni, WT-saline vs. Tg*NTRK3*-saline $p = 4.3E-05$, WT-saline vs. WT-NT3 $p = 0.05$, Tg*NTRK3*-saline vs. Tg*NTRK3*-NT3 $p = 0.0008$). As a conclusion

local NT3 administration in the mPFC during the consolidation phase of contextual fear extinction is sufficient to rescues Tg*NTRK3* impaired extinction memory.

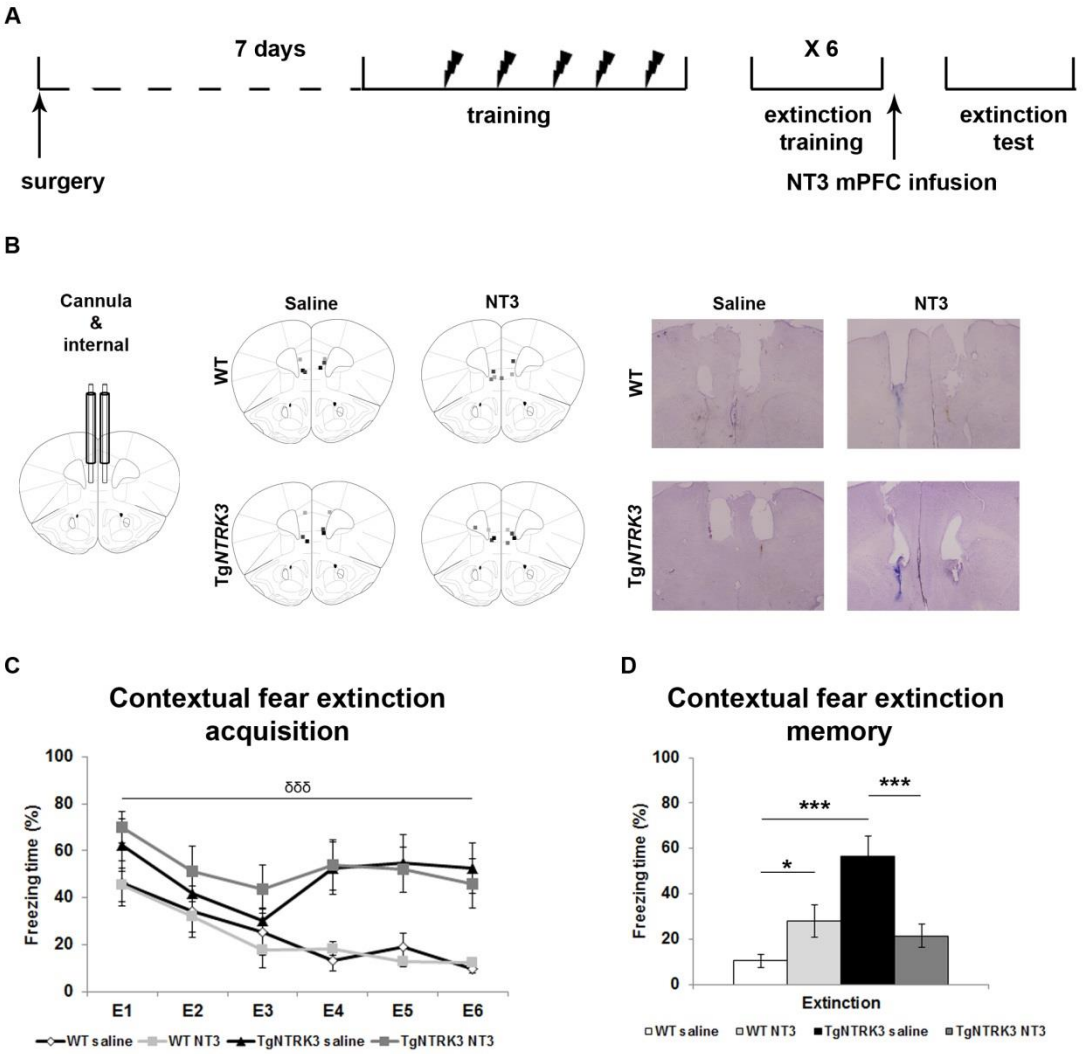

Figure 19: Effect of intra-mPFC NT3 administration on contextual fear extinction memory of WT and Tg*NTRK3* mice. (A) Schematic representation of the stereotaxic surgical cannulae implantation and pure contextual fear extinction paradigm with intra-mPFC infusion of NT3 after the extinction training session. (B) Schematic representation of the brain region in which cannulae were implanted and infusions were performed (stereotaxic coordinates: AP = 2.0, ML = ± 0.50, DV = - 1.2), real injection sites for each animal used in the experiment infused with either saline or NT3 (WT-saline n = 8, WT- NT3 0.75µg/side n = 10, Tg*NTRK3*-saline n = 7 and Tg*NTRK3*-NT3 0.75µg/side n = 9) and representative histological sections. (C-D) Effect of intra-mPFC NT3 infusion on contextual fear extinction memory. Percentage of freezing time spent by WT and Tg*NTRK3* mice treated with either saline or NT3 during the (C) extinction training

session and (D) extinction test. We used repeated measures 2-way ANOVA with sessions as within subject factor and genotype and treatment as between subject factors (extinction training session) and 2-way ANOVA with genotype and treatment as factors (extinction test), *post hoc* Bonferroni upon genotype x treatment interaction * $p < 0.05$, *** $p < 0.001$ $^{\delta\delta\delta}$ genotype effect $p < 0.001$. NT3, neurotrophin type 3; mPFC, medial prefrontal cortex; WT, wild type; Tg*NTRK3*, transgenic mice overexpressing *NTRK3*.

DISCUSSION

5. DISCUSSION

PAND is a severe anxiety disorder in which one of the most ancestral emotions, fear, is disturbed, representing the hallmark feature. The high impact that this psychiatric condition has on society (Alonso, Angermeyer et al. 2004) demands a deep understanding of the pathophysiological mechanism/s underlying abnormal and exaggerated fear responses, such as the strong association of fearful experiences (panic attack) with the environment in which they occur (Bouton, Mineka et al. 2001; Lissek, Powers et al. 2005). To this aim, this thesis focuses in studying fear memory in a transgenic mouse model validated for PAND, Tg*NTRK3* mice (Dierssen, Gratacos et al. 2006). The Tg*NTRK3* is the only genetic model that has shown validity for modelling PAND and is thus a powerful tool to validate therapies. Although it only contains one gene and PAND is plausibly polygenic, the fact that it recapitulates most of the symptoms/signs recognized in a PAND subpopulation contributes to our knowledge of general brain malfunction in aspects that are possibly shared across PAND subtypes.

We have investigated how Tg*NTRK3* mouse model of PAND might recapitulate the enhanced fear memory processes seen in patients and studied the involvement of different fear circuit brain regions. Further, we explored which phases of the fear learning process are disturbed and to what extent the observed phenotype is due to a direct involvement of the neurotrophin system and/or other systems, such as the glutamatergic and GABAergic.

5.1 Fear learning and memory in Tg*NTRK3* mice

Fear learning and memory are hypothesized to be central features in PAND development and establishment. As previously described, the problem in these patients is the enhanced valence that PAND patients attribute to aversive experiences, such as panic attacks.

In the present study, using different fear-conditioning paradigms we found that Tg*NTRK3* mice show specifically enhanced contextual and temporal fear

memories, both hippocampal-dependent learning processes. When submitted to a pure contextual fear-conditioning paradigm, Tg*NTRK3* mice showed normal acquisition of contextual fear memory (Figure 1B), but increased fear memory expression, both at 24 hours and one week after training (Figure 1C). A first consideration regarding this phenotype is that it is relevant for the clinical features of PAND, in which daily life aversive experiences, which occur in a multitude of associated stimuli, involving multiple senses and overall defined as context, can trigger a panic attack. Our pure contextual paradigm resembles experimentally this situation since mice learn to associate a unique multi-sensorial context (CS) to the experience of an aversive foot shock (US). The observed enhanced fear memory represents thus a relevant phenotype revealing the neurobiological mechanism that might contribute to PAND.

Recently Parsons and colleagues (2013) have pointed to the importance of the dissection of the different cognitive phases and components that regulate fear memories in fear disorders. In our pure contextual fear conditioning paradigm, Tg*NTRK3* mice did not show any difference as compared to WT during the acquisition session, but they showed enhanced memory 24 hours later, suggesting that the cognitive component affected in these mice might be the consolidation, that time window after conditioning required for the stabilization of a persistent memory. This finding is relevant because it might set up the basis for a time-sensitive therapeutical window during which fear memory can be tackled and rescued in PAND, for instance by administering pharmacological treatments that block consolidation after the experience of a panic attack in a given context. With this scope we further investigated which could be the mechanism responsible for the consolidation of contextual fear memory in the Tg*NTRK3* mice (see below), taking into account that this process is controlled by multiple molecular and cellular mechanisms (Johansen, Cain et al. 2011; Orsini and Maren 2012).

The enhanced contextual fear memory in Tg*NTRK3* mice has been observed not only 24 hours after training but is also maintained one week later. The persistence of this phenotype over a relatively long period of time may have different not mutually exclusive explanations. It could be merely the result of a

stronger consolidation process or it could reflect incapacity of Tg*NTRK3* mice to extinguish acquired fear memories. Extinction is an active re-learning process that requires several expositions to the CS (context), without reinforcement by the US. In fact, in a pure contextual fear extinction paradigm, Tg*NTRK3* mice showed a marked resistance to extinction, both during the acquisition (Figure 4C) and retrieval (Figure 4D) phases. This phenotype is of relevance as deficits in fear extinction have also been observed in other anxiety disorders, such as post-traumatic stress disorder (PTSD) and PAND (Blechert, Michael et al. 2007; Michael, Blechert et al. 2007). The understanding of the underlying neural mechanism of such resistance to extinction of fear memories would definitively open new strategies for therapy.

In the extinction protocol that we used (classically known as within-session extinction) the CS has been presented to the mice six times separated one hour each, within one single day of extinction training. This protocol has failed to induce in the Tg*NTRK3* mice a reduction in their freezing response, contrary to WT littermates and when tested for extinction memory, 24 hours later, they continued to show high fear response. The possibility to treat panic disorder patients with exposure therapies to those stimuli associated to aversive experiences in combination with pharmacological treatment (Otto, Tolin et al. 2010) open the question of which would be the best protocol in term of exposure times and windows of time between each exposition, in order the obtain a more efficient extinction of fear.

In this respect, we recently demonstrated that in a different behavioural paradigm Tg*NTRK3* mice did show a normal extinction of contextual fear memory (Santos, D'Amico et al. 2013). Using a between-session extinction paradigm, mice where submitted to an extinction training procedure consisting of six re-expositions to the CS, each 24 hours apart from the other. These long extinction inter trial intervals allow for multiple consolidation events during the extinction learning process and probably activate some nodes of the fear circuit that we found to be perturbed (see next sections 5.2). Again these data suggest that consolidation phases within fear learning and memory processes are crucial time windows to tackle in a therapy perspective. Interestingly, treating

rats with the metabotropic glutamate receptor type 7 (mGLUR7) agonist AMN082, showed a differential effect in the two extinction paradigms, impairing the within-session extinction, but facilitating the between-session extinction (Toth, Dietz et al. 2012). This example suggests that when considering for a pharmacological treatment in combination with cognitive therapy it is highly relevant both the exposure schedule and the timing of drug administration, for a successful induction of extinction.

One particular feature of PAND panic attacks is the fact that they often are unpredictable, occurring “out of the blue”. The unpredictability of the panic attacks in PAND patients leads to a persistent worry about the occurrence of other attacks and to anticipatory anxiety (Helbig-Lang, Lang et al. 2012). In fact, patients showed increase anxiety to unpredictable but not to predictable aversive stimuli (Grillon, Lissek et al. 2008). In order to test the Tg*NTRK3* mice for their susceptibility to unpredictable aversive experiences we used the trace fear conditioning paradigm. In this paradigm the CS (tone) is separated from the US (shock) by a trace (interval of time). The trace between the CS and the US makes the association less predictable and can recapitulate the trait described in patients. We found that temporal fear memory is enhanced in Tg*NTRK3* mice when tested 24 hours after training (Figure 2C), confirming that the unpredictability of an aversive event induces a stronger response, as occurs in PAND patients. Interestingly, also in this paradigm the freezing response of Tg*NTRK3* mice during the acquisition phase was not different than WT littermates, confirming that the enhanced fear phenotype is caused by a stronger consolidation of memory. However, contrary to what we observed for contextual fear memory, Tg*NTRK3* mice showed normal temporal fear memory one week after the training session. It is described that in this type of paradigm the temporal-dependence is a hippocampus property and that the long-term memory seems more dependent on cortical brain areas (Quinn, Ma et al. 2008).

Finally, we demonstrated that the enhanced fear memory of Tg*NTRK3* mice is not a general phenomenon, but specific for those memories that rely on an

intact hippocampal function. In fact, when Tg*NTRK3* mice were submitted to the delay / cue fear conditioning paradigm, in which a tone is immediately followed by the shock, WT and Tg*NTRK3* littermates showed similar freezing response when tested for memory 24 hours after conditioning (Figure 3B). This process involves mostly the auditory cortex and the thalamus to process information to the amygdala. In this paradigm, the occurrence of the aversive foot shock is highly predictable by the cue and a possible explanation for the normal fear memory observed in the Tg*NTRK3* mice is that, indeed, they are normally sensitive to a well predictive aversive experience.

The specificity of the enhanced hippocampal-dependent fear memories observed in the Tg*NTRK3* mouse model of PAND opened the basis for a potential key role of this brain region in the disorder. Similar hippocampal-dependent fear memories are indeed central aspects of PAND altered fear spectrum (see introduction section 1.4). Interestingly other genetic mouse models also show alteration in specific types or phases of fear memories. Nevertheless the Tg*NTRK3* phenotype seems specific for PAND, as it points to a specificity of perturbed hippocampal dysfunction. For instance the *5-htt -/-* mice (knockout for the serotonin transporter) present impaired contextual fear conditioning (Muller, Morelli et al. 2011) and impaired extinction recall (Wellman, Izquierdo et al. 2007). In this case, since both contextual learning conditioning and extinction are impaired, *5-htt -/-* mice would be better defined as a model with impaired contextual memory, rather than enhanced fear memories, as Tg*NTRK3* mice.

Modifications in other genes of the neurotrophin family have also been proposed to be involved in fear-related disorders (Zhang, Benedek et al. 2013). In accordance, mice heterozygous for the brain-derived neurotrophic factor gene (*Bdnf)* show impaired contextual fear memory, but intact cue fear learning (Liu, Lyons et al. 2004), suggesting that as *NTRK3*, *Bdnf* is involved specifically in contextual fear memory, but not in cue fear.

The glutamatergic and GABAergic systems as key players in learning and memory events, have undoubtedly a role in the fear process. Mice

overexpressing the NMDA glutamate receptor subunit 2B gene (*Nr2b*) gene show enhanced contextual and cue fear memory and faster extinction (Tang, Shimizu et al. 1999). Differentially to *Ntrk3*, the Nr2b subunit seems to be involved in fear learning in general and not specifically to one type of fear memory. Interestingly, a possible common mechanism might be involved in the Tg*NTRK3* mice altered fear, as they also showed increased hippocampal expression levels of Nr1 and Nr2b unphosphorilated (inactive), but not phosphorilated (active) isoforms (Sahun, Delgado-Garcia et al. 2007). However, our pharmacological study with ifenprodil (Figure 15E), together with the fact that phosphorilated Nr2B isoforms are not changed in the TgNTRK3 mice, suggest a more complex mechanism, involving also the GABAergic system (see below 5.3). Finally, confirmations that the GABA system play a role in fear memories come, as an example, from mice lacking the alpha 4 subunit of GABA receptor ($GABA_A$), resulting in a less inhibited network, which show enhanced trace and contextual fear memories (Moore, Cushman et al. 2010). This piece of data supports the hypothesis that in our model a lack of inhibitory control over the fear neural networks.

Although these and other models might share some phenotypic alterations with the Tg*NTRK3* fear memory phenotype, each of these has its own specificity regarding types of fear memory alterations, and none of it recapitulate exactly the pattern observed in the Tg*NTRK3* model, so we can conclude that the genetic overexpression of TrkC plays a unique role in the enhancement of contextual and temporal fear memory, not affecting cue fear memory.

5.2 Fear circuit activation in Tg*NTRK3* mice

In order to elucidate which brain area and the specific sub-region involved, we performed a series of c-FOS quantitative analysis in the hippocampus, amygdala and prefrontal cortex during consolidation of contextual fear memory and consolidation of extinction of contextual fear memory.

One first interesting finding is observed in the hippocampus, a key structure in contextual fear memory expression and extinction (Corcoran, Desmond et al. 2005). The detailed analysis of c-FOS expression, revealed a stronger activation of hippocampal CA1py excitatory neurons during fear conditioning (Figure 7D, 10C) which was also sustained during extinction (Figure 9D, 11E). This cellular phenotype was observed specifically in the ventral portion of the hippocampus and not in the dorsal part (Figure 7B, 9B). In accordance, the expression of another early gene commonly used as a marker of neuronal activation, *Arc*, Inoue and colleagues (2005) reported a shift from dorsal to ventral hippocampus in the activation of CA1 pyramidal neurons, upon contextual fear conditioning in rats. However, the specificity of the dorsal vs. ventral in fear learning seems to depend also on the phase of the learning process and involves the glutamatergic system: pre-training NMDA receptor antagonism in the dorsal or ventral hippocampus impaired the acquisition of both trace and contextual conditioning, while pre-testing NMDA receptor antagonism in the ventral, but not dorsal, impaired the expression of previously-acquired trace and contextual fear conditioning (Czerniawski, Ree et al. 2012).
Several other studies have demonstrated that the dorsal hippocampus is particularly involved in spatial learning rather than emotional learning. As an example spatial learning in the Morris water maze paradigm is strongly impaired upon lesions of the dorsal part, while similar lesions in the ventral do not affect spatial performance (Moser, Moser et al. 1993; Pothuizen, Zhang et al. 2004). A more recent study has confirmed the role of the dorsal hippocampus on spatial function. Here, muscimol temporary inactivation of the dorsal, but not ventral, dramatically impaired performance in the spatial reinforced alternation task, while the same inactivation of the ventral, but not the dorsal, attenuated the acquisition and expression of trace fear conditioning (Czerniawski, Yoon et al. 2009). In our recent work we showed that Tg*NTRK3* mice present a mild impairment in spatial learning in the Morris water maze paradigm (Santos, D'Amico et al. 2013), so possibly during a spatial learning process Tg*NTRK3* mice might reveal some specific alteration in the dorsal portion of the hippocampus that could underlie this phenotype.

Besides the dorsal vs. ventral differences observed, we also found that the overactivation maps predominantly to the CA1py layer and not in other hippocampal layers. Here localize the output neurons that receive terminals from the CA3py and communicate with other brain structures in the fear circuit (see introduction section 1.2). An important consideration and limitation of our analysis is the impossibility to distinguish which subpopulations of pyramidal neurons are overactivated. It has been shown that CA1 pyramidal neurons project to the amygdala (Pitkanen, Pikkarainen et al. 2000; Herry, Ciocchi et al. 2008), to the mPFC (Vertes 2006) and some of them also project simultaneously to both structures through an axonal bifurcation located near their soma (Ishikawa and Nakamura 2006). The final result of fear might be substantially different depending on which CA1py neuronal subpopulation would be overactivated in each given time point of the fear learning process, therefore the identification of those types in the Tg*NTRK3* mice would probably define a “pyramidal-specific” neuronal type/s responsible for exaggerated fear memories in PAND.

All together these evidences let allow us to conclude that the ventral CA1 hippocampal subregion of Tg*NTRK3* mice is strongly engaged in the enhanced fear memory and thus might play a central role in fear memories in PAND.

The amygdala is by excellence the core brain region of fear (Maren and Quirk 2004), required for both unconditioned and conditioned fear (Davis 2000) and an exquisite control of the intra-amygdalar circuit activation is required for conditioning and extinction of fear memories (Herry, Ciocchi et al. 2008; Herry, Ferraguti et al. 2010). Upon fear conditioning Tg*NTRK3* mice show higher activation of the CeM (Figure 7G) which was maintained also upon extinction (Figure 9G), in opposite to what occurred in WT littermates. Being CeM neurons the final output neurons gating fear responses (LeDoux, Iwata et al. 1988; Ciocchi, Herry et al. 2010), the pattern of activation in Tg*NTRK3* mice is in agreement with the higher and not extinguished contextual fear memory. Moreover, CeM neurons account also for innate fear responses (Nanda, Qi et al. 2008) that are intrinsic and do not require any learning process. In the

Tg*NTRK3* model we found a higher number of c-FOS positive neurons in the CeM also in naïve conditions (Figure 7G, 9G), as compared to WT naïve mice. Interestingly our group have previously shown that Tg*NTRK3* mice present an increased innate fear/panic-like phenotype in the mouse defence test battery paradigm; in fact, exposing these mice to a natural predator induced multiple signs of panic-like and fear responses, such as higher flight and freezing behaviour (Dierssen, Gratacos et al. 2006). Thus the enhanced activation that we found in the CeM in naïve conditions might be extrapolated as a potential mechanism also responsible for high innate fear in the Tg*NTRK3* mice.

It has been described that multiple entry routes of information converge into several amygdala nuclei, such as the BA, LA and ITCs (Sotres-Bayon, Bush et al. 2004; Herry, Ciocchi et al. 2008). And although the classical view have pointed the LA and BA nuclei as the principal entrance of inputs, in both these regions we found no changes in activation neither in WT nor in Tg*NTRK3* mice upon contextual fear conditioning and extinction, as compared to the naïve condition (Figure 7F, 9F). In the basolateral nuclei, Tg*NTRK3* mice also did not show differences to WT in the number of Thy1-YFP/c-FOS double positive, excitatory neurons, upon contextual fear conditioning or extinction (Figure 10E, 11G), although it has been recently proposed that this neuronal subpopulation may mediate inhibition of fear (Jasnow, Ehrlich et al. 2013). This apparently incongruent result can be due to compensatory activation and/or de-activation processes of other specific neuronal populations within the basolateral amygdala. In fact, context-dependent expression and extinction of fear in this region is tightly controlled by excitatory and several different types of inhibitory neurons (Ehrlich, Humeau et al. 2009), working together in a complex and dynamic network (Vlachos, Herry et al. 2011).
An interestingly pattern of activation was observed in the lITC and mITC of the amygdala, in which Tg*NTRK3* mice showed a marked reduced activation both during conditioning and extinction (Figure 7F, G, 9F, G). The intercalated clusters are islands of GABAergic neurons that regulate the intra-amygdala circuit (Likhtik, Popa et al. 2008) and have been defined as the “off switch” for

the amygdala and therefore for fear (Quirk and Mueller 2008). Thus, the dramatic reduced activation observed in Tg*NTRK3* mice in contextual fear learning processes suggests a lack of ITC-mediated switch off mechanism for pathological fear, in line with the exaggerated contextual fear memory found. However, a more complex level of function within the ITCs has been recently described; in fact during different states of fear learning and memory specific cell type in the ITCs are differentially involved (Busti, Geracitano et al. 2011). In our c-FOS experiments we have not accounted for the specificity or sub-localization of inactivated ITC neurons and for this reason future studies should pursue the identification of which ITC cell type is lacking activation in Tg*NTRK3* mice during fear memory. The identification of defined subpopulations will ultimately define targeted therapeutical strategies to re-activate ITCs neurons and rescue exaggerated PAND fear memories.

Finally, we also analysed the neuronal activation pattern of the mPFC, which revealed another piece of the complex fear circuit activation properties in Tg*NTRK3* mice. We found that Tg*NTRK3* mice show a reduced activation of AC and IL cortices upon fear conditioning (Figure 7H), where it seems not to play any essential role in WT littermates, as they did not show any differences as compared to naive WT. More interestingly a lack of activation in both PL and IL cortices during extinction was observed in Tg*NTRK3* (Figure 9H) that is in accordance with their impaired ability to extinguish fear memory. Moreover, when quantified the number of activated excitatory neurons (Thy1-YFP/c-FOS), upon extinction, we found a selective reduced activation of excitatory neurons in the IL, but not in the PL (Figure 11C). All together these results led us to conclude that a lack of modulatory control of the mPFC over the amygdala is responsible of the impaired extinction phenotype of Tg*NTRK3* mice. Neurons in the IL are necessary for proper extinction (Milad and Quirk 2002) and stimulation of PL and IL regions showed differential involvement of the two mPFC sub-regions, being the first responsible of exciting amygdala output, via the BA, and the second responsible of inhibiting amygdala output, via mITC (Vidal-Gonzalez, Vidal-Gonzalez et al. 2006). In addition it has been shown that

chemical stimulation of the IL activates c-FOS expression in ITC neurons (Berretta, Pantazopoulos et al. 2005), so the reduced activation that we observed in the IL of Tg*NTRK3* mice might directly be responsible for the reduced activation of the ITCs in the amygdala (see above). In spite of the important role that the activation pattern represents in modulating fear processing, there are other levels of regulation, as plasticity mechanisms involving protein synthesis (Santini, Ge et al. 2004) and neurotrophic factors (Peters, Dieppa-Perea et al. 2010), that we will discuss later in this thesis.

As a general conclusion, one possible mechanism for the Tg*NTRK3* enhanced contextual fear memories is the disruption of the hippocampus – amygdala - mPFC brain fear circuit. Our data are in accordance with a model that we propose as the pathological fear circuit in PAND mouse model Tg*NTRK3* (Figure 1). Here, 1) the higher and sustained overactivation of the ventral CA1 pyramidal neurons in the hippocampus is contributing to an “over-evaluation” of contextual input, 2) the lack of activation of IL neurons in the mPFC leads to an impaired ability to properly activate ITC-mediated local inhibitory control. These two lastly induce 3) the sustained overactivation of fear generating neurons in the CeM amygdala and thus exaggerated fear memories, observed in pathological fear.

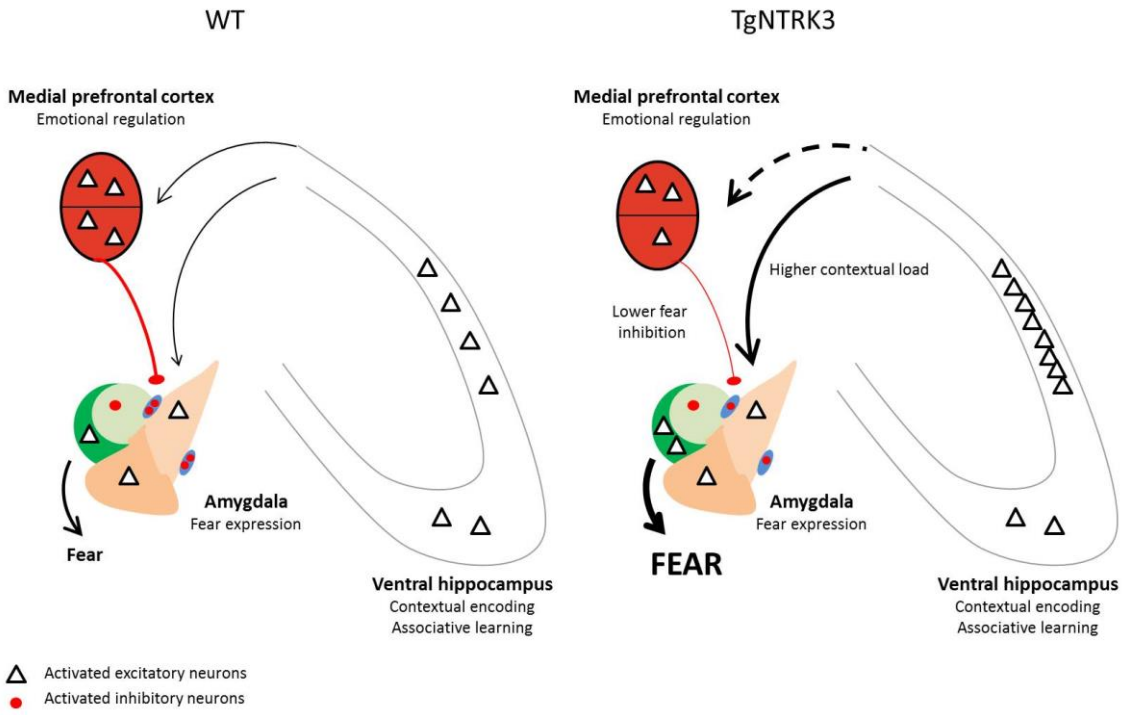

Figure 1: Model of WT and Tg*NTRK3* hippocampus – amygdala - medial prefrontal cortex fear circuit. Schematic representation of the most consistent findings in the quantitative analysis of neuronal activation pattern, during contextual fear memories. Briefly Tg*NTRK3* mice exaggerated contextual fear memories are underlined by the overactivation of ventral CA1 pyramidal neurons in the hippocampus and lack of activation of excitatory neurons in the IL region of the medial prefrontal cortex. This probably results to the reduced activation of neurons in the ITCs, finally contributing to a reduced inhibitory control within the amygdala circuit and therefore overactivation of fear output neurons in the CeM favoring exaggerated fear.

5.3 Hippocampal excitatory vs. inhibitory dysbalance in Tg*NTRK3* mice, a potential system for therapy

The hyperactivated ventral hippocampal pattern found in Tg*NTRK3* mice during fear memory processing has pointed to mainly a local dysfunction in the CA1py region, where mostly excitatory neurons are located and these put into the fear circuit context-related information. As this input could play a central role in the establishment and maintenance of exaggerated fear memory in PAND, we further investigated through which mechanism this output hippocampal neurons are functionally over-engaged when fear memory is formed.

We previously described (see introduction section 1.6) that the intra-hippocampal network is well organized and multiple types of excitatory and

inhibitory neurons are distributed in specific layers and sub-regions. One possibility is that Tg*NTRK3* mice may present altered numbers of specific cell types, disrupting the network, and to test for this we quantified the number of excitatory and inhibitory cell in different hippocampus sub-regions. We found that Tg*NTRK3* mice do not show any alteration in the densities of Thy1-YFP positive pyramidal excitatory neurons (Figure 12D), nor GAD 65/67 positive inhibitory interneurons (Figure 13D) in any of the hippocampal layers analysed, concluding that the hippocampal enhanced activation is not due to an altered number of either excitatory or inhibitory neurons.

The complexity of the hippocampal functions is greatly increased by the contribution of multitude subpopulations of neurons. As an example CA1py neurons are innervated by at least twelve distinct types of GABAergic neurons, belonging to four different groups, accordingly to the markers they express, and their axonal arborization density and projections sites (Klausberger 2009). One possibility is that in the Tg*NTRK3* mice some of these GABAergic subpopulations might be altered in their density or functionality and thus contributing to a lack of inhibitory control over CA1py activity.

Not only the number of neurons accounts for the proper neuronal transmission and network, but also a fine tuning is mediated by the billions of synaptic contacts that are created and continuously changed in response to the environment. CA1py neurons show a well-conserved cellular architecture with their pyramidal-shaped soma located in the pyramidal layer, from where they send two principal braches of dendrites that then arborize in opposite directions to make their synaptic contacts: one into the *stratum oriens* (basal dendrites) and the other deeply immersed in the *stratum radiatum* (apical dendrites), with some branches reaching the more ventral layer *lacunosum moleculare* (Spruston 2008). In the *stratum radiatum* apical dendrites, CA1 pyramidal neurons receive excitatory inputs from CA3py neurons and inhibitory inputs from local GABAergic neurons (Megias, Emri et al. 2001). We quantified the balance between these two types of inputs into CA1srad layer and Tg*NTRK3* mice showed higher VGLUT1 / VGAT puncta ratio, as compared to WT littermates (Figure 14B). This enhanced glutamatergic/GABAergic presynaptic

balance represents an evidence for an increased excitable CA3-CA1 circuit in Tg*NTRK3* mice, in a context of a normal density of excitatory and inhibitory neuronal populations. The VGLUT1 / VGAT increased ratio in Tg*NTRK3* mice is mainly due to a higher number of VGLUT1 puncta, suggesting a possible increased density of glutamatergic synapses. Nevertheless, our analysis of the number of excitatory synapses showed that these are not different as compared to WT littermates (VGLUT1 / PSD95 double positive puncta, Figure 14D). Our results suggest that the CA1py neurons of the ventral hippocampus of Tg*NTRK3* mice are probably receiving an exaggerated excitatory load. In accordance to this conclusion we can argue that this overexcited region in the brain of these mice is also functionally responding to a much stronger level upon stimulation, in fact, previous studies showed that *in vivo* high frequency stimulation in the CA3 resulted in an enhanced and longer sustained LTP measured in CA1 (Sahun, Delgado-Garcia et al. 2007). Importantly, the genetic depletion of VGLUT1 in Vglut1$^{-/-}$ mice cause a severe impairment in the excitatory glutamatergic neurotransmission (Fremeau, Kam et al. 2004) and impaired LTP in the CA1 region in Vglut1$^{+/-}$ heterozygous mice (Balschun, Moechars et al. 2010), along with enhanced anxiety and impaired memory (Tordera, Totterdell et al. 2007; Balschun, Moechars et al. 2010), suggesting an essential role for VGLUT1 not only in synaptic vesicles refilling with glutamate, but also glutamatergic transmission efficiency and glutamatergic-dependent behaviours. Interestingly the effect of NT3 and TrkC receptor in balancing the excitatory/inhibitory systems has been shown. NT3 induces glutamate release at the synaptic cleft and favours the maturation of excitatory synapses rather than inhibitory ones (Collin, Vicario-Abejon et al. 2001), arguing in favour that NT3-TrkC effects on the glutamatergic system could directly cause the hyperexcitability of Tg*NTRK3* mice.

On the other hand also the reduction in GABAergic load has similar effect; for instance a reduction in VGAT puncta density in the CA1srad and CA3srad, without changes in the number of inhibitory synapses, has been described in mice knockout for the neuroligin type 2 (Nlgn2$^{-/-}$) which correlates with an increased anxiety-like phenotype (Blundell, Tabuchi et al. 2009). Potentially in

the Tg*NTRK3* overexcitable hippocampus, both the glutamatergic and GABAergic systems are mutually deregulated. In fact, in total hippocampal protein extracts Tg*NTRK3* mice show increased levels of VGLUT1 and reduced levels of GAD 65/67 (Santos, D'Amico et al. 2013), confirming that both systems are affected and the final result is a dysbalanced excitatory to inhibitory network.

Our next experiment involved the use of pharmacological tools to either block the glutamatergic or enhance the GABAergic systems, with the aim of re-balancing the system in Tg*NTRK3* mice and finally rescue contextual fear memory to normal levels. We demonstrate that peripheral treatment with tiagabine, a GABA reuptake inhibitor, but not with ifenprodil, a selective NR2B antagonist, during the consolidation phase was able to rescue the enhanced contextual fear memory (Figure 15E, 16E). This could be explained by the fact that the glutamatergic system in Tg*NTRK3* mice is saturated (Sahun, Delgado-Garcia et al. 2007) and requires a break, such a potentiation of the GABAergic system, to function properly. The positive effect observed with the tiagabine on one side confirm to efficiently re-balance the TgNTRK3 excitatory / inhibitory system and, on the other, from a clinical point of view, suggests that tackling and enhancing the GABAergic system can efficiently treat pathological fear. This is not a novel concept as benzodiazepines, GABA receptor agonists, such as lorezapam (Schweizer, Pohl et al. 1990), alprazolam (Verster and Volkerts 2004) and clonazepam (Rosenbaum 2004) have been proved to be effective in treating PAND patients. These are high-potency benzodiazepines found to be effective and with a rapid onset of action; however their use has important secondary effects, such as dependence, rebound anxiety and general memory impairment (Chouinard 2004) and benzodiazepines long-term treatments caused rapid tolerance and symptoms of withdrawal upon cessation (Bateson 2002). The mechanism of action of tiagabine might open new hopes in PAND and other fear-related disorders, because, contrary to benzodiazepines that act as external and mimetic GABA, tiagabine acts on the GABAergic system by blocking synaptic GABA reuptake, thereby increasing endogenous GABA levels at synapses (Zwanzger and Rupprecht 2004) and allowing for its normal, but

extended, inhibitory transmission. This could probably represent better efficacy and less or smaller side effects. In animals tiagabine was able to reduce anxiety in the open-field and elevated plus maze task (Schmitt and Hiemke 1999) and, more importantly, in PAND patients it reduces anxiety, agoraphobia and the number of panic attacks with only light and reversible side effects, such as dizziness and sedation during the first day of treatments (Zwanzger and Rupprecht 2005). In a subsequent study the same group showed that, although tiagabine treatment did not differ from placebo in general clinical symptoms in PAND patients, it clearly reduced their sensitivity to experimentally induced panic attack (Zwanzger, Eser et al. 2009; Zwanzger, Eser et al. 2009). Finally the beneficial effects of tiagabine treatments have been shown also for other anxiety disorders such as generalized anxiety disorder (Rosenthal 2003) or posttraumatic stress disorder (Taylor 2003), suggesting that it might also modulate common features shared by anxiety disorders.

Our results demonstrate that tiagabine can rescue the contextual fear memory in the Tg*NTRK3* mice and add a new level of potential benefit for the treatment of not only panic attacks and anxiety in PAND, but also for the formation of those exaggerated fear memories, that reinforce the progression of the disorder. Moreover our finding that tiagabine administration in the ventral hippocampus was able to reduce contextual fear memory both in WT and Tg*NTRK3* mice (Figure 17D) confirmed a key mechanism for the consolidation of this type of memory and suggest that the enhancement of synaptic GABAergic transmission is sufficient to re-balance the overexcitable system in the TgNTRK3 mice. In addition it highlights the role of this region in pathological fear formation and PAND.

5.4 Neurotrophin 3 in fear: a source of plasticity in Tg*NTRK3*

The enhanced fear memory found in Tg*NTRK3* mice is the result of the impaired fear circuit network through a hippocampal unbalanced glutamatergic/GABAergic system. As previously discussed (see Introduction, section 1.6) fear learning and memory processes are dependent on proper

plasticity mechanisms in key brain regions for adapting behavioural responses to the given environmental condition. The association of the *NTRK3* gene to fear disorders confirmed that change in its expression, regulation and functioning are, indeed, involved in the affected ability of patients to respond in a "normal" way to threating events and stimuli (Gratacos, Nadal et al. 2001; Armengol, Gratacos et al. 2002; Muinos-Gimeno, Guidi et al. 2009). However, the direct involvement of TrkC and its high-affinity ligand, NT3, during specific phases of fear learning processes have not been consistently investigated.

A large body of evidence suggested the direct involvement of the cognate neurotrophin BDNF and its high affinity receptor, TrkB, in mPFC plasticity underlying extinction. In fact, local administration of BDNF in the mPFC is sufficient to induce extinction of fear via modulation of the glutamatergic system (Peters, Dieppa-Perea et al. 2010). In this thesis we characterized the expression levels of TrkC and NT3 during contextual fear learning processes in WT and Tg*NTRK3* mice, with the goal to investigate their function on fear memories and their direct involvement in Tg*NTRK3* enhanced and extinction-resistance contextual fear. We found that both WT and Tg*NTRK3* mice showed a marked increase in TrkC, full length isoform, upon contextual fear training, which is even more notorious upon extinction training in the mPFC (Figure 18C, E), suggesting that it may actually be required in mPFC plasticity mechanisms needed for proper fear learning. Although similar increases have been found in the two genotypes, the behavioural freezing response was much different, so the expression level of the TrkC only, during fear learning processes, does not explain the impaired extinction phenotype in Tg*NTRK3* mice. Our analysis from hippocampal extracts showed that after contextual fear extinction Tg*NTRK3* mice show reduced TrkC levels, as compared to WT animals in the same condition, suggesting a potential inhibitory regulation of the expression of TrkC in the hippocampus which might be responsible for the impaired extinction. One possible explanation for this could be found in the possible requirement of activation of the TrkC receptor, mediated by the binding of the NT3. In fact, this leads to intracellular pathways like the MAPK to be turned on (English, Pearson et al. 1999; Pearson, Robinson et al. 2001; Reichardt 2006) and then

contributing to fear conditioning and fear extinction (Atkins, Selcher et al. 1998; Fischer, Radulovic et al. 2007; Tronson, Schrick et al. 2009). Therefore for a complete understanding of the process we also quantified NT3 protein levels during fear memories. In the hippocampus, we detected no differences in NT3 content between WT and Tg*NTRK3* mice in naïve, fear conditioning or fear extinction processes (Figure 18F). In the mPFC, Tg*NTRK3* mice showed reduced NT3 levels after contextual fear conditioning and extinction trainings, contrary to WT littermates (Figure 18G), suggesting that the impaired fear extinction phenotype may be caused by a local deficit in NT3 in the mPFC. Our results of the hippocampus showed, opposite to the mPFC, normal levels of NT3, within the context of reduced TrkC levels during extinction, in Tg*NTRK3* mice. Although affecting different players, the possible final effect could be equally a lack of the NT3-TrkC mediated plasticity required for contextual fear extinction learning, in the hippocampus – medial prefrontal cortex fear circuit in Tg*NTRK3* mice, supporting the impaired ability to extinguish contextual fear memory. This hypothesis is highly plausible because, similarly, the BDNF that facilitate extinction by acting in the mPFC is derived and released here by long range projecting hippocampal neurons (Peters, Dieppa-Perea et al. 2010). At hippocampus-mPFC synapses, BDNF binds to postsynaptic TrkB receptors, leading to activation of intracellular downstream pathways, including the MAPK, shared with NT3-TrkC. In addition BDNF binds as well to presynaptic TrkB receptors favouring glutamate release from the projectin neuron, thus activating the excitatory neurotransmission in the target neuron (Andero and Ressler 2012). Common intracellular pathways and influences of the glutamatergic system, shared by the BDNF-TrkB and NT3-TrkC cognate neurotrophic systems, suggest a common involvement of the two in fear memories. This opens the possibility to supply and/or stimulate neurotrophin levels to rescue the extinction phenotype.

As a proof of the concept that Tg*NTRK3* impaired contextual fear extinction might be dependent on a lack of NT3 mediated plasticity in the mPFC, we administered locally rhNT3 in the mPFC of WT and Tg*NTRK3* mice immediately after contextual fear extinction training (Figure 19A). We found that local

infusion of NT3 in the IL portion of the mPFC induced a complete rescue of the freezing response in Tg*NTRK3* mice, as tested for extinction memory twenty-four hours after infusion (Figure 19D). In Tg*NTRK3* mice the local NT3 supplemented the ineffective training procedure (Figure 19C). Interestingly in WT littermates, the local application of NT3 in the mPFC in animals that already well acquired extinction learning during training (Figure 19C), caused a mild impairment of contextual fear extinction (Figure 19D), suggesting that NT3 levels must be tightly regulated and that in excess will preclude extinction learning. These results showed for the first time the involvement of NT3 in extinction learning, but also suggest that this effect occurs in a dose-dependent manner, coordinated with the expression and regulation of its receptor TrkC, at least in the mPFC brain region.

In light of these results and of the involvement of other neurotrophins, such as BDNF, in fear extinction, future studies should be focused in understanding at a finer level how physiologically the mPFC receive and/or produce neurotrophins, how neurons respond to these growth factors, by regulating the expression of their receptors and how the activation of these pathways finally activate neurons to input “fear off” signals in the brain fear circuit.

CONCLUSIONS

6. CONCLUSIONS

This doctoral thesis highlights novel and clinically relevant findings in the understanding of a key mechanism responsible for altered fear memory processes in PAND, using the unique genetic model of PAND, the Tg*NTRK3* mice:

- The genetic overexpression of *NTRK3* gene in Tg*NTRK3* mice induces enhanced and extinction resistant hippocampal-dependent fear memory, that point towards a key role of the hippocampus in pathological fear in PAND.
- The enhanced contextual fear memories observed in Tg*NTRK3* mice are associated to a perturbed activation pattern of the fear circuit. Overactivation of the hippocampus along with a lack of activation of the mPFC, converge into an impaired activation of local inhibitory controls within the amygdala circuit, that finally results in exaggerated fear responses.
- Hippocampal hyperexcitabity underlies the enhanced consolidation of contextual fear memory in Tg*NTRK3* mice. Pharmacological potentiation of synaptic GABAergic neurotransmission is sufficient to re-balance the system and rescue the fear memory phenotype.
- The impaired consolidation of extinction of contextual fear memory in Tg*NTRK3* mice is associated to an impaired functioning of the NT3-TrkC system in the hippocampus – mPFC crosstalk. Exogenous administration of NT3 in the mPFC rescues the impaired extinction phenotype.

The genetic predisposing factors to develop PAND might favour the establishment and maintenance of disturbed cognitive phenotypes, related to aversive experiences, such as fear memories. These may be underlined by alteration in those neural mechanisms, physiologically controlling fear and cognition, such as unregulated neuronal excitability and impaired neuronal plasticity in the brain fear circuit.

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ANNEXES

8. ANNEXES

I. Abbreviations and Acronyms

- AC: anterior cingulate
- ACC: anterior cingulate cortex
- AMPA: A-Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic Acid receptor
- Amy: amygdala
- B6.Cg-Tg(Thy1-YFPH)2Jrs/J: transgenic mouse line expressing YFP under *Thy1* promoter
- BA: basal
- BDNF: brain-derived neurotrophic factor
- BLA: basolateral
- BSA: bovine serum albumin
- CA1: cornu ammonis area 1
- CA3: cornu ammonis area 3
- CeL: centro lateral
- CeM: centro medial
- CNS: central nervous system
- CS: conditioned stimulus
- CTX: context
- DAB: 3,3'-diaminobenzidine
- DAPI: 4',6-diamidino-2-phenylindole
- DG: dentate gyrus
- dHP: dorsal hippocampus
- DNA: Deoxyribonucleic acid
- EC: *entorhinal cortex*
- ELISA: enzyme linked immuno-sorbant assay
- ERK: extracellular signal-regulated kinase
- FBS: fetal bovine serum
- FC: fear conditioning
- FExt: fear extinction

- GABA: γ aminobutyric acid
- Gad 65/67: glutamic acid decarboxylase isoforms of 65 and 67 KDa
- GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
- H_2O_2: hydrogen peroxide
- HCl: hydrochloridic acid
- HCL: hydrogen chloride
- HP: hippocampus
- IL: infralimbic
- i.p.: intra peritoneal
- IP3-PKC: inositide 3-phosphate-protein kinase C
- ITI: inter-trial interval
- JNK: c-Jun N-terminal kinases
- LA: lateral
- lITC: lateral intercalated cell cluster
- MAPK: mitogen-activated protein kinase
- MetOH: methyl alcohol
- mITC: medial intercalated cell cluster
- mPFC: medial prefrontal cortex
- MRI: magnetic resonance imaging
- mRNA: messenger ribonucleic acid
- Na_3VO_4: sodium orthovanadate
- NaF: sodium fluoride
- NaOH: sodium hydroxide
- NF-$_k$B: nuclear factor k activated B cells
- NGF: nerve growth factor
- NMDA: N-methyl-D-aspartate glutamate receptor
- NP40: nonyl phenoxypolyethoxylethanol
- Nr2b: NMDA glutamate receptor subunit 2B gene
- NT3: neurotrophin 3
- NT4/5: neurotrophin 4/5
- *NTRK3*: neurotrophin tyrosine kinase receptor type 3 gene

- OR: *stratum oriens*
- P75 NTR: p75 neurotrophin receptor
- PAND: panic disorder
- PBS: phosphate buffer saline
- PDGF: platelet-derived growth factor
- PFA: paraformaldehyde
- PI3K: phosphatidylinositide 3 kinase
- PI3K-Akt: phosphatidylinositide 3 kinase-protein kinase B
- PL: prelimbic
- PLC-γ: phosphoinositide phospholipace C type γ
- PMSF: phenylmethylsulfonyl fluoride
- PSD95: postsynaptic density protein 95
- PVC: polyvinyl chloride
- Py: *stratum pyramidale*
- Rad: *stratum radiatum*
- rpm: revolutions per minute
- RT: room temperature
- s.c.: sub cutaneous
- SSC: saline-sodium citrate buffer
- Tg*NTRK3*: transgenic mice overexpressing the human *NTRK3* gene
- *Thy1*: thymus cell antigen 1
- Thy1-YFP/*NTRK3:* transgenic mice expressing yellow fluorescent protein under *Thy1* promoter and overexpressing the human *NTRK3* gene
- Thy1-YFP/WT*:* transgenic mice expressing yellow fluorescent under *Thy1* promoter
- Trk: tyrosine kinase receptors
- TrkA: tyrosine kinase receptor type 1
- TrkB: tyrosine kinase receptor type 2
- TrkC: tyrosine kinase receptor type 3
- US: unconditioned stimulus
- VGAT: vesicular GABA transporter

- VGLUT1: vesicular glutamate transporter type 1
- vHP: ventral hippocampus
- WT: wild type mice
- YFP: yellow fluorescent protein

II. Development of a phobic avoidance behavioural model

An important proportion of PAND patients might develop several forms of phobic behaviours (DSM-V 2013). Some patients are classified as PAND patients with agoraphobia and these have the tendency to avoid open spaces as they induce a lower control of possible attacks. A more common type of phobic reaction in PAND patients is the phobic avoidance of those contexts in which a panic attack occur (see introduction). In this section we describe our attempt to develop a behavioural paradigm to study phobic avoidance in mice, by using the conditioned place aversion test and panicogenics as conditioning factors. Sevaral different drugs have been proposed to induce panic attack in PAND patients and animals, however not all patients responde to all drugs and thus represent subtypes of PAND with various sensitivity to drug-induced panic attack. For instance one of the most used drug to induce a panic attack is sodium lactate, that induce attacks in patients (Johnson, Truitt et al. 2008) and panic-like symptoms in animals (Bergold, Pinkhasova et al. 2009). Another widely used drug is caffeine, in fact at high doses of caffeine in PAND patients induce increased anxiety and panic attacks (reviewed in (Vilarim, Rocha Araujo et al. 2011). Moreover it is widely known that stressfull experiences may produce panic-like symptoms in PAND patients. Previous studies of our laboratory have shown that Tg*NTRK3* mice are responsive to panicogenic drugs, such as caffeine and sodium lactate (Sahun, Gallego et al. 2007) and are highly susceptible to stress (Amador-Arjona, Delgado-Morales et al. 2010). Therefore we have used these pharmacological and behavioural challenges in order to induce conditioned place aversion, a model to study phobic avoidance in the Tg*NTRK3* mice.

Phobic avoidance experiments were performed in the Automated Place Preference apparatus (Panlab Harvard Apparatus, Barcelona, Spain) consisted of a chamber/ context A (three white walls and one dark transparent; black smooth floor) and a chamber/ context B (three black walls and one transparent; white rough floor), separated by a small corridor and the chambers isolated

from the corridor by removable doors. The floors lie over weight sensors, connected to an electrical module, to detect the presence of the animal. The time spent in each compartment of the apparatus was automatically recorded by using the PPCWin v2.0 software (Panlab Harvard Apparatus).

The behavioural paradigm consisted in a pre-test session (day 1) in which mice were placed in the apparatus with open doors and the time spent in each compartment was recorded for 15 min. Twenty-four hours later the conditioning phase started: on day 2 the animals were intraperitoneal (i.p.) injected with saline and placed in one of the two chambers with the door closed and on day 3 they were injected with the conditioning drug (panicogenic) and placed in the other chamber with the door closed (counterbalanced for day of conditioning and chamber of conditioning). On day 4 animals were tested in the same apparatus with open doors and the time spent in each compartment was recorded for 15 min. Control groups for each genotype received two saline injections in both contexts and days of conditioning. In the immobilization experiment (see section II.Experiment 7) animals were conditioned by leaving them free in one of the chamber on day 2 and immobilized in a tube on day 3.

The primary measure used as analysis of place conditioning is the time spent in the drug-context during the pre-test and the test sessions. In order to analyze differences in conditioned place aversion phenotype the measure taken in account was the Change In Preference (CIP) index = (TT – TPT)/ (TT + TPT) = (Time Test – Time Pre Test)/ (Time Test + Time Pre Test) and averaged per group (WT_Saline, WT_Drug, Tg*NTRK3*_Saline, Tg*NTRK3*_Drug); moreover for each panicogenic drug a doses curve experiment has been performed in only WT animals to determine the effective dose.

Statistical analysis were performed with repeated measure one-way ANOVA for time in zone (pre-test vs. test) and one-way ANOVA for CIP index in the doses curve experiments. Repeated measure two-way ANOVA with genotype and treatment as between-subject factors and time in zone (pre-test vs. test) as within-subject factors and two-way ANOVA for CIP index in all the experiments with both WT ang Tg*NTRK3* mice.

The following drugs have been used to induce conditioned place aversion in different experimental conditions:

Experiment 1: Caffeine doses curve in B6SJL mice

WT animals were divided in ascending doses of caffeine (C07504, Sigma), an adenosine receptor type 2a antagonist, and were injected with the doses of 10 mg/Kg (N = 7), 30 mg/Kg (N = 8), 40 mg/Kg (N = 8) and 60 mg/Kg (N = 8) and compared to a saline group (N = 8) in one session of one hour of conditioning (day 2: one hour saline in chamber A, day 3: one hour caffeine/ saline in chamber B, counterbalanced) (Fig. II.1A). No significant diffrences in the time spent in the caffeine zone has been osberved between groups, resulting in no Change In Preference indexes (Fig. II.1B, C).

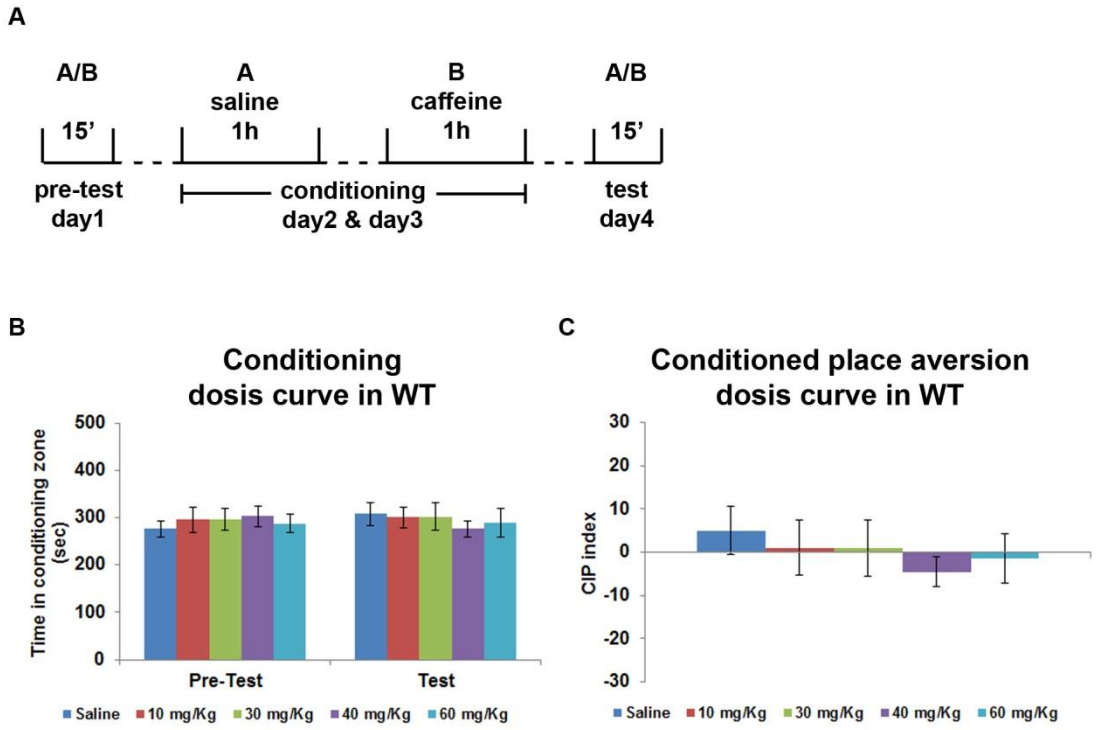

Figure II.1: Caffeine doses curve conditioned place aversion in WT animals. (A) Schematic representation of the conditioned place aversion behavioural paradigm with one conditioning session composed of one-hour saline in A/ caffeine in B exposure. (B) Graphic showing the time spent in the conditioning zone per group during the pre-test and test sessions and (C) Change In Preference index, CIP = (Time Test – Time Pre Test)/ (Time Test + Time Pre Test). Statistical analysis: repeated measure one-way ANOVA for time in zone (pre-test vs. test) and one-way ANOVA for CIP index. A, chamber A; B, chamber B, A/B, opened place preference apparatus; WT, wild type; CIP, change in preference index.

Experiment 2: Caffeine induced phobic avoidance, one conditioning day

WT and Tg*NTRK3* animals were conditioned in one session of one hour of conditioning (day 2: one hour saline in chamber A, day 3: one hour caffeine/ saline in chamber B, counterbalanced) with caffeine at 30 mg/ Kg dose and tested for caffeine-induced place aversion, as compared to saline conditioned group (Fig. II.2A). WT_saline (N = 5), WT_ caffeine 30 mg/Kg (N = 3), Tg*NTRK3*_saline (N = 4) and Tg*NTRK3*_caffeine 30 mg/Kg (N = 5). No significant diffrences in the time spent in the caffeine zone has been osberved between groups, resulting in no Change In Preference indexes (Figure II.2B, C).

Experiment 3: Caffeine induced phobic avoidance, two conditioning days

WT and Tg*NTRK3* animals were conditioned in two sessions of one hour of conditioning (day 2 and 4: one hour saline in chamber A, day 3 and 5: one hour caffeine/ saline in chamber B, counterbalanced) with caffeine at 30 mg/ Kg dose and tested for caffeine-induced place aversion, as compared to saline conditioned group (Fig. II.2D). WT_saline (N = 4), WT_ caffeine 30 mg/Kg (N = 5), Tg*NTRK3*_saline (N = 4) and Tg*NTRK3*_caffeine 30 mg/Kg (N = 4). No significant diffrences in the time spent in the caffeine zone has been osberved between groups, resulting in no Change In Preference indexes (Figure II.2E, F).

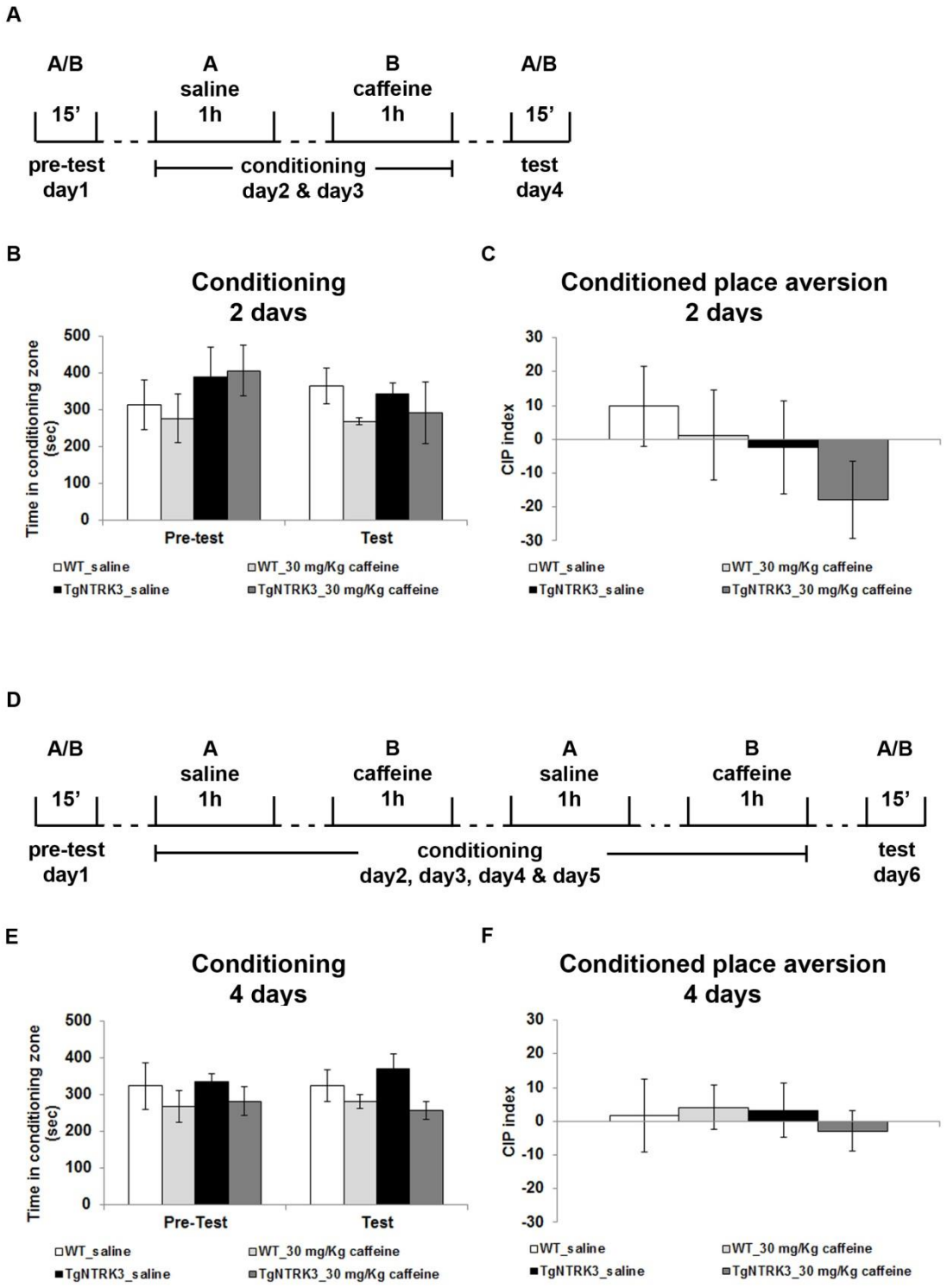

Figure II.2: Caffeine conditioned place aversion in WT and Tg*NTRK3* animals. Schematic representation of the conditioned place aversion behavioural paradigm with (A) one conditioning session composed of one-hour saline in A/ caffeine in B exposure or (D) two conditioning sessions composed of one-hour saline in A/ caffeine in B exposures. (B, F) Graphic showing the

time spent in the conditioning zone per group during the pre-test and test sessions and (C, F) Change In Preference index, CIP = (Time Test – Time Pre Test)/ (Time Test + Time Pre Test). Statistical analysis: repeated measure two-way ANOVA with genotype and treatment as between-subject factors and time in zone (pre-test vs. test) as within-subject factors and two-way ANOVA for CIP index. A, chamber A; B, chamber B, A/B, opened place preference apparatus; WT, wild type; Tg*NTRK3*, transgenic mice overexpressing TrkC; CIP, change in preference index.

Experiment 4: Sodium lactate doses curve in B6SJL mice.

WT animals were divided in ascending doses of sodium lactate, SL (71720, Fluka Analytic, Sigma), the sodium salt of lactic acid, C3H5NaO3, metabolized in the brain with the final production of CO2. Mice were injected with the doses of 140 mg/ Kg (N = 4), 280 mg/ Kg (N = 5), 560 mg/ Kg (N = 5) and compared to a saline group (N = 4) in one session of one hour of conditioning (day 2: one hour saline in chamber A, day 3: one hour SL/ saline in chamber B, counterbalanced) (Fig. II3.A). No significant diffrences in the time spent in the SL zone has been osberved between groups, resulting in no Change In Preference indexes (Figure II3B, C).

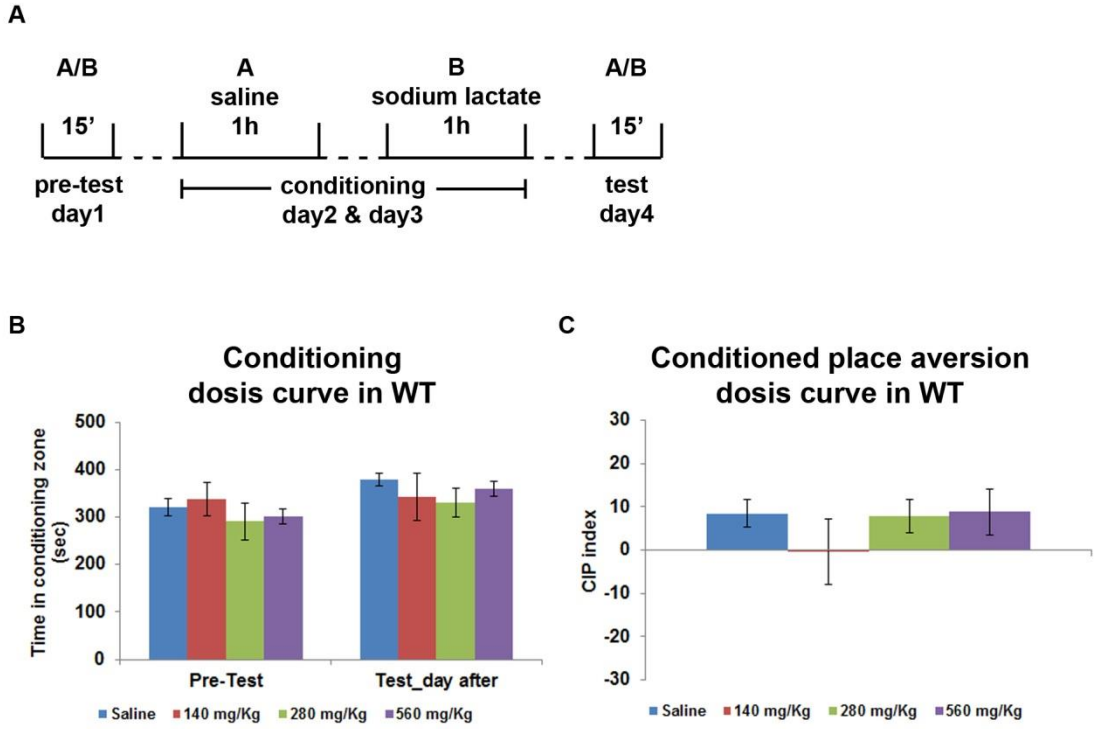

Figure II.3: Sodium lactate (SL) doses curve conditioned place aversion in WT animals. (A) Schematic representation of the conditioned place aversion behavioural paradigm with one

conditioning session composed of one-hour saline in A/ SL in B exposure. (B) Graphic showing the time spent in the conditioning zone per group during the pre-test and test sessions and (C) Change In Preference index, CIP = (Time Test – Time Pre Test)/ (Time Test + Time Pre Test). Statistical analysis: repeated measure one-way ANOVA for time in zone (pre-test vs. test) and one-way ANOVA for CIP index. A, chamber A; B, chamber B, A/B, opened place preference apparatus; WT, wild type; CIP, change in preference index.

Experiment 5: Sodium lactate induced phobic avoidance, one conditioning day, one hour lasting

WT and Tg*NTRK3* animals were conditioned in one session of one hour of conditioning (day 2: one hour saline in chamber A, day 3: one hour SL/ saline in chamber B, counterbalanced) with SL at 280 mg/ Kg dose and tested for SL-induced place aversion, as compared to saline conditioned group (Fig. II.4A). WT_saline (N = 9), WT_ SL 280 mg/Kg (N = 9), Tg*NTRK3*_saline (N = 7) and Tg*NTRK3*_ SL 280 mg/Kg (N = 6). No significant diffrences in the time spent in the caffeine zone has been osberved between groups, resulting in no Change In Preference indexes (Figure II.4B, C).

Experiment 6: Sodium lactate induced phobic avoidance, one conditioning day, ten minutes lasting

WT and Tg*NTRK3* animals were conditioned in one session of ten min of conditioning (day 2: ten min saline in chamber A, day 3: ten min SL/ saline in chamber B, counterbalanced) with SL at 280 mg/ Kg dose and tested for SL-induced place aversion, as compared to saline conditioned group (Fig. II.4D). WT_saline, WT_ SL 280 mg/Kg, Tg*NTRK3*_saline and Tg*NTRK3*_ SL 280 mg/Kg (N = 3/ group). No significant diffrences in the time spent in the caffeine zone has been osberved between groups, resulting in no Change In Preference indexes (Figure II.4E, F).

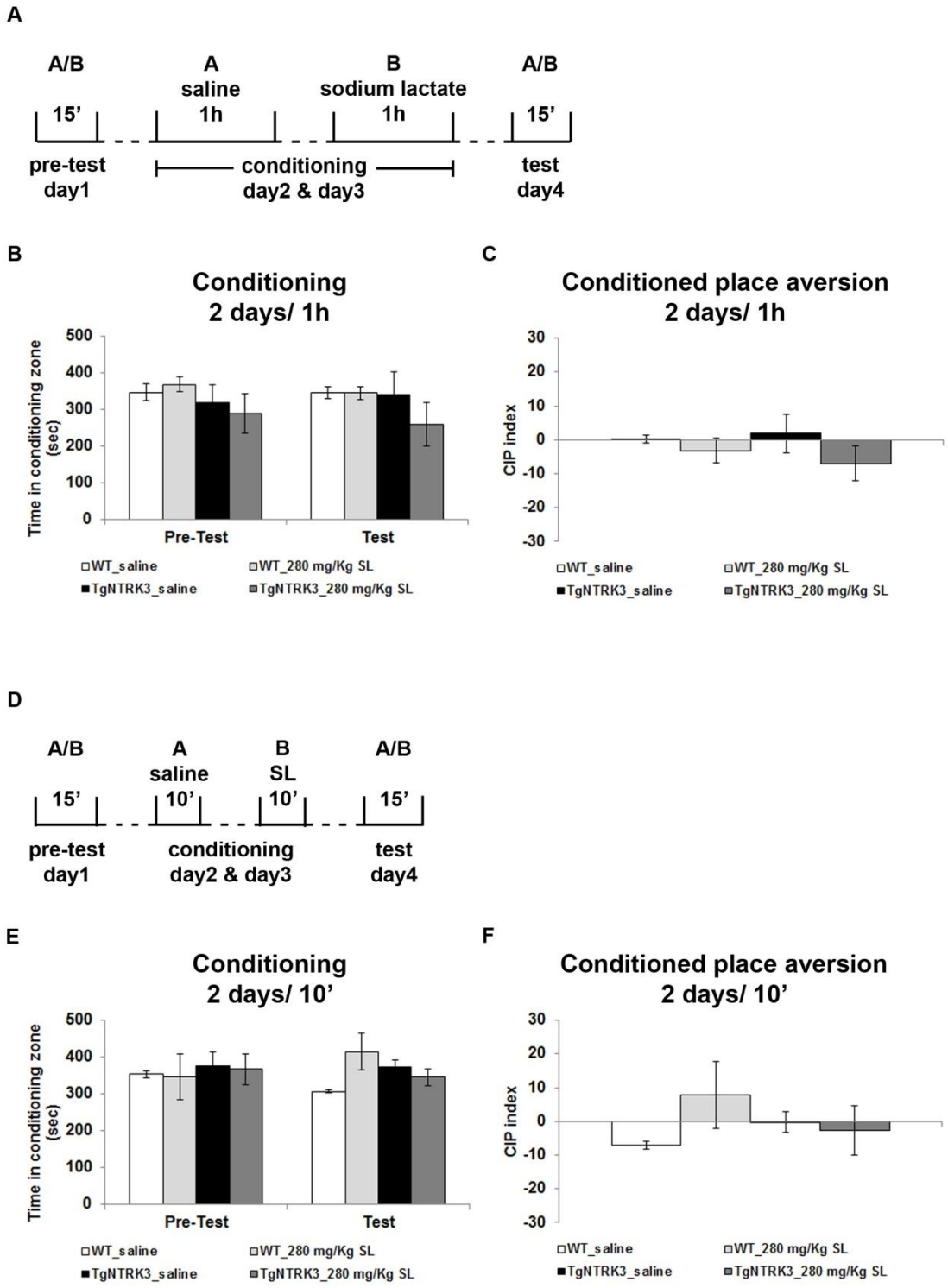

Figure II.4: SL conditioned place aversion in WT and Tg*NTRK3* animals. Schematic representation of the conditioned place aversion behavioural paradigm with (A) one conditioning session composed of one-hour saline in A/ SL in B exposure or (D) one conditioning sessions composed of 10-min saline in A/ SL in B exposures. (B, F) Graphic showing the time spent in the conditioning zone per group during the pre-test and test sessions and (C, F) Change In

Preference index, CIP = (Time Test – Time Pre Test)/ (Time Test + Time Pre Test). Statistical analysis: repeated measure two-way ANOVA with genotype and treatment as between-subject factors and time in zone (pre-test vs. test) as within-subject factors and two-way ANOVA for CIP index. A, chamber A; B, chamber B, A/B, opened place preference apparatus; WT, wild type; Tg*NTRK3*, transgenic mice overexpressing TrkC; CIP, change in preference index.

Experiment 7: Immobilization induced phobic avoidance, one conditioning day, one hour lasting

WT and Tg*NTRK3* animals (N = 12) were conditioned in one session of one hour of conditioning (day 2: one hour free in chamber A, day 3: one hour immobilized, IMO, in chamber B, counterbalanced) using immobilization as a panic-like inducing stimulus and tested for IMO-induced place aversion (Fig. II.5A). No significant diffrences in the time spent in the IMO zone has been osberved between genotypes, resulting in no Change In Preference indexes (Figure II.5E, F).

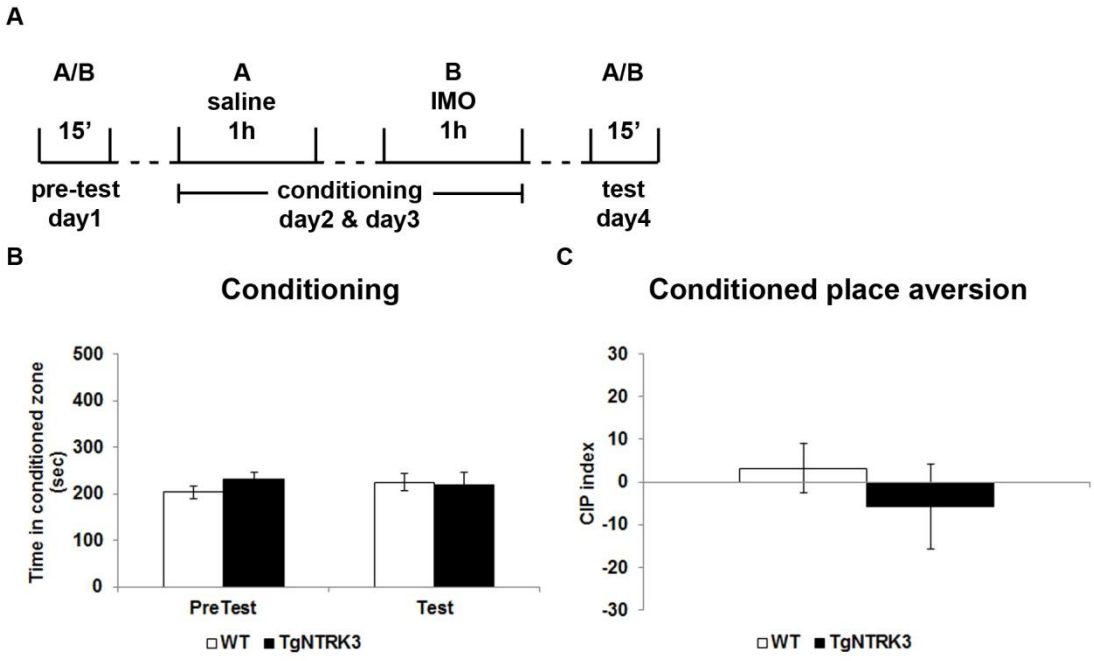

Figure II.5: Immobilization (IMO) conditioned place aversion in WT and Tg*NTRK3* animals. (A) Schematic representation of the conditioned place aversion behavioural paradigm with one conditioning session composed of one-hour free in A/ IMO in B exposure. (B) Graphic showing the time spent in the conditioning zone per group during the pre-test and test sessions and (C) Change In Preference index, CIP = (Time Test – Time Pre Test)/ (Time Test + Time Pre Test). Statistical analysis: repeated measure two-way ANOVA with genotype and time in zone (pre-

test vs. test) as within-subject factors and Student's t-test for CIP index. A, chamber A; B, chamber B, A/B, opened place preference apparatus; WT, wild type; Tg*NTRK3*, transgenic mice overexpressing TrkC; CIP, change in preference index.

As a general conclusion of this section we can argue that neither sodium lactate nor caffeine or immobilization induced conditioned place aversion in the Tg*NTRK3* animals. However, being this a optimization attempt to develop a novel behavioural paradigm, we cannot exclude the possibility that these negative results might be the consequence of purely our experimental conditions. Thus future attention should be also pointed to use other types of apparatus and environmental conditions and a more broad analysis of other panicogenic stimuli.

III. Publications and communications

Publications

Santos M *, **D'Amico D ***, Spadoni O, Amador-Arjona A, Stork O, Dierssen M. Hippocampal hyperexcitability underlies enhanced fear memories in Tg*NTRK3*, a panic disorder mouse model. J Neurosci 33(38): 15259-15271. * Both authors equally contributed to this work.

Santos M, **D'Amico D**, Dierssen M. Panic disorder: insights from human neuroimaging and genetic studies and the contribution of animal models. Review article in preparation (peer reviewed invitation by Genes Brain & Behavior).

D'Amico D *, Santos M *, Dierssen M. NT3 induced extinction of fear memories in Tg*NTRK3*, a panic disorder mouse model. Manuscript In preparation. * Both authors equally contributed to this work.

Communications

Santos M, **D'Amico D**, Dierssen M. Hippocampal hyperexcitability underlies enhanced fear memories in Tg*NTRK3* panic disorder mouse model. Oral presentation by Santos M. at XIII Portughese Society for Neuroscience meeting, Luso – Portugal, 2013.

D'Amico D, Santos M, Dierssen M. Abnormal fear circuit activation upon in vivo TrkC overexpression. Poster presentation at 42nd SfN's annual meting, New Orleans – USA, 2012.

D'Amico D, Santos M, Dierssen M. Abnormal fear circuit activation upon in vivo TrkC overexpression. Poster presentation at 8th FENS Forum of European Neuroscience, Barcelona – Spain, 2012.

Santos M, **D'Amico D**, Dierssen M. In vivo TrK C overexpression induces hyperexcitability and strengthens hippocampus-dependent fear memories: molecular mechanism underneath. Poster presentation by Santos M. at 8th FENS Forum of European Neuroscience, Barcelona – Spain, 2012.

D'Amico D: Member of the students committee of the 8th FENS Forum of European Neuroscience, Barcelona – Spain, 2012.

Santos M, **D'Amico D**, Vegue M, Dierssen M. Hippocampal overexcitability in a mouse model of panic disorder Poster presentation by Santos M. at 40th SfN's annual meting, San Diego – USA, 2010.

D'Amico D, Santos M, Vegue M, Dierssen M. Hippocampal neural circuit for fear memory in Tg*NTRK3*, a mouse model of panic disorder**.** Poster presentation at EMBO|EMBL Symposium: Structure and Function of Neural Circuits, EMBL Heidelberg – Germany, 2010.

D'Amico D, Santos M, Dierssen M. Hippocampal alterations associated with fear memories in Tg*NTRK3* mouse, a model of panic disorde**.** Poster presentation at EMBL Workshop on Translating Behaviour: Bridging Clinical and Animal Model Research, EMBL Heidelberg – Germany, 2009

D'Amico D *, Spadoni O *, Santos M, Dierssen M. Characterization of the hippocampal microcircuit in a mouse model of panic disorder**.** Oral presentation at Fifth Cajal Winter Conference – Sociedad Espaňola de NeroCiencia (SENC) Neuronal Generation, Growth and Degeneration, Benasque, Spain, 2009. * Both authors equally contributed to this work.

Santos M, D'Amico D, Spadoni O, Amador-Arjona A, Stork O, Dierssen M. Hippocampal hyperexcitability underlies enhanced fear memories in TgNTRK3, a panic disorder mouse model. J Neurosci. 2013 Sep 18; 33(38): 15259-71. DOI: 10.1523/JNEUROSCI.2161-13.2013

Neurobiology of Disease

Hippocampal Hyperexcitability Underlies Enhanced Fear Memories in Tg*NTRK3*, a Panic Disorder Mouse Model

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Panic attacks are a hallmark in panic disorder (PAND). During the panic attack, a strong association with the surrounding context is established suggesting that the hippocampus may be critically involved in the pathophysiology of PAND, given its role in contextual processing. We previously showed that variation in the expression of the neurotrophin tyrosine kinase receptor type 3 (*NTRK3*) in both PAND patients and a transgenic mouse model (Tg*NTRK3*) may have a role in PAND pathophysiology.

Our study examines hippocampal function and activation of the brain fear network in Tg*NTRK3* mice. Tg*NTRK3* mice showed increased fear memories accompanied by impaired extinction, congruent with an altered activation pattern of the amygdala—hippocampus—medial prefrontal cortex fear circuit. Moreover, Tg*NTRK3* mice also showed an unbalanced excitation-to-inhibition ratio in the hippocampal cornu ammonis 3 (CA3)–CA1 subcircuit toward hyperexcitability. The resulting hippocampal hyperexcitability underlies the enhanced fear memories, as supported by the efficacy of tiagabine, a GABA reuptake inhibitor, to rescue fear response.

The fearful phenotype appears to be the result of hippocampal hyperexcitability and aberrant fear circuit activation. We conclude that *NTRK3* plays a role in PAND by regulating hippocampus-dependent fear memories.

Introduction

Human studies have shown that the neurotrophin tyrosine kinase receptor type 3 (*NTRK3*) gene, encoding the high-affinity tropomyosin receptor kinase C (TrkC) receptor, may contribute to the genetic susceptibility to psychiatric disorders such as panic disorder (PAND), obsessive-compulsive disorder, or schizophrenia (Gratacòs et al., 2001; Armengol et al., 2002; Alonso et al., 2008; Muiños-Gimeno et al., 2009; Otnaess et al., 2009). Reported *NTRK3* genetic variations are predicted to change TrkC mRNA expression levels (Gratacòs et al., 2001; Armengol et al., 2002; Muiños-Gimeno et al., 2009) and alter synaptic plasticity through a change of local trophic support. Indeed, neurotrophins regulate synaptic function and plasticity (for review, see McAllister et al., 1999; Huang and Reichardt, 2001) and may thus be involved in CNS dysfunction (Minichiello et al., 1999; Sahún et al., 2007; Otnaess et al., 2009; Hong et al., 2011).

PAND is one of the most common anxiety disorders (American Psychiatric Association, 2000; Roy-Byrne et al., 2006) with a lifetime prevalence of 1.1% to 3.7% (Goodwin et al., 2005; Kessler et al., 2006; Skapinakis et al., 2011), characterized by the occurrence of panic attacks and disturbances in the fear circuit. In PAND, the context in which panic attack occurs, is a determinant factor in the establishment of the disorder. In this respect, the hippocampus is a key brain structure contributing to the context dependency of fear expression and extinction (Ext; Corcoran et al., 2005; Sierra-Mercado et al., 2011). PAND patients show structural and functional alterations in prefrontal cortex, hippocampus, and amygdala brain regions (Massana et al., 2003a,b; for review, see Charney, 2003) with abnormal frontolimbic activation patterns (Maren and Quirk, 2004; Herry et al., 2008; Sierra-Mercado et al., 2011). TrkC receptor is highly expressed in these brain regions (Ernfors et al., 1992; Krause et al., 2008), and, therefore, even small disturbances in its expression levels might have a strong impact on their function. Several years ago, we validated a transgenic mouse overexpressing human *NTRK3* as a model of PAND (Dierssen et al., 2006). Tg*NTRK3* mice show the following: (1) face validity with increased panic-like reaction and heightened anxiety; (2) construct validity with increased numbers of noradrenergic neurons in the locus ceruleus; and (3) predictive validity as they respond positively to diazepam treatment in the elevated plus maze paradigm (Dierssen et al., 2006). More-

Received May 22, 2013; revised July 23, 2013; accepted Aug. 13, 2013.

Author contributions: M.D. designed research; M.S., D.D., O. Spadoni, A.A.-A., and O. Stork performed research; M.S., D.D., A.A.-A., and M.D. analyzed data; M.S., D.D., and M.D. wrote the paper.

The laboratory of M.D. is supported by Departament d'Universitats, Recerca i Societat de la Informació (Grups consolidats 09 2009SGR1313). This work was supported by Grants SAF2010-16427, SAF2007-31093-E, and FIS (PI 082038); Marató TV3; the Jerome Lejeune, Koplowitz, and Areces Foundations; and the European Union (LSHG-CT-2006-037627; CureFXS ERare-EU/FIS PS09102673). The Centro de Investigación Biomédica en Red de Enfermedades Raras is an initiative of the Instituto de Salud Carlos III. M.S. was supported by Fundação para a Ciência e Tecnologica (Portugal) with a postdoctoral fellowship, and D.D. was supported by a La Caixa International PhD Programme fellowship.

*M.S. and D.D. contributed equally to this work.

The authors declare no competing financial interests.

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DOI:10.1523/JNEUROSCI.2161-13.2013

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over, Tg*NTRK3* mice also show strongly enhanced and sustained hippocampal long-term potentiation (LTP; Sahún et al., 2007).

In this study, we hypothesized that variation in the expression levels of TrkC in fear network brain regions, particularly in the hippocampus, contribute to PAND context-related emotional learning dysfunction. Here, we show that TrkC-overexpressing mice present enhanced fear memories and altered activation of the brain fear circuit. Furthermore, we showed that by targeting the GABAergic system we were able to rescue the enhanced fear memory exhibited by Tg*NTRK3* mice to normal wild-type (WT) levels.

Materials and Methods

Animals

We used young adult (2–4 months old) male Tg*NTRK3* mice overexpressing the human *NTRK3* gene and previously validated as a model of PAND (Dierssen et al., 2006). A double-transgenic line was created by crossing Tg*NTRK3* with transgenic B6.Cg-Tg(Thy1-YFPH)2Jrs/J mice (The Jackson Laboratory; Feng et al., 2000) named Thy1-YFPH:*NTRK3* in which the Thy-1 promoter directs the expression of the yellow fluorescent protein (YFP) to a subpopulation of excitatory neurons. The WT littermates served as controls for all of the experiments. Genotypes were determined by PCR according to protocol established in the laboratory (Dierssen et al., 2006) or, in the case of the fluorescent strains, provided by The Jackson Laboratory. All animal procedures were approved by the local ethics committee, and met the guidelines of the local and European regulations (European Union directive no. 86/609; European Union decree 2001-486).

Pure contextual fear-conditioning paradigm

The fear-conditioning and fear extinction experimental paradigms were performed in a fear-conditioning apparatus (StartFear, Panlab Harvard Apparatus) and freezing behavior, defined as lack of movement other than breathing for at least 2 s, was automatically recorded using commercial software (FREEZING, Panlab Harvard Apparatus). Freezing response is a reliable measure of conditioned fear in rodents.

Mice were paired an initially neutral context [conditioned stimulus (CS)] with an aversive electric footshock [unconditioned stimulus (US)]. On the first day, animals were placed in the testing chamber for a 3 min habituation session. Twenty-four hours later, mice were trained in the same chamber in a 5 min session composed of 2 min of exploration followed by five US presentations (footshock: 2 s, 0.2 mA), separated by a variable intertrial interval (ITI; 15–60 s; Fig. 1*A*); mice remained in the chamber for 30 s following the last US. Freezing behavior was measured for 15 s after each shock. Twenty-four hours and 1 week later, mice were tested in the same chamber for contextual fear memory by scoring freezing time in a 2 min session.

Trace fear-conditioning paradigm

Mice were trained to associate a neutral tone (CS) with an aversive electric footshock (US), separated by a trace (Fig. 1*D*). On the first day, a 3 min habituation session was performed. Twenty-four hours later, mice were trained in the same chamber in a 7 min session composed of 2 min of exploration, followed by five CS–US presentations (CS: 10 s, 100 dB, 2000 Hz; trace: 18 s; US: 2 s, 0.2 mA), separated by a variable ITI (from 15 to 60 s). Freezing behavior was measured for 18 s of each trace interval. Twenty-four hours and 1 week after the training session, mice were tested for trace fear memory in the same chamber but with different floor texture, wall color and pattern, odor, and light intensity, without US presentation, by scoring freezing time.

Within-session pure contextual fear extinction, reinstatement, and contextual generalization

Within-session fear extinction was performed 24 h after pure contextual fear-conditioning training (see above) and consisted of one single session with six trials [extinction session 1 (E1) to E6], each one lasting 2 min and separated from the next by a 1 h interval. The following day, mice were tested for fear memory extinction in the same context for 2 min. After the extinction test, mice received one US and the reinstatement of the extinguished memory was analyzed by measuring freezing time in a 2 min session. For contextual generalization assessment, 24 h after the reinstatement test mice were placed in the same chamber but with different floor texture, wall color and pattern, odor, and light intensity, and freezing was measured for 2 min.

Between-session pure contextual fear extinction

Mice were first fear conditioned in a pure contextual paradigm (see above) and 24 h later were subjected to the between-session extinction protocol. For 6 consecutive days (E1–E6), WT and Tg*NTRK3* mice were presented with the CS without reinforcement by the US in a 2 min session, and freezing time was measured.

Water maze paradigm

Mice were trained in a water maze paradigm (diameter: pool, 170 cm^2; platform, 8 cm^2; Vorhees and Williams, 2006), and their movement was detected with a video tracking system (SMART, Panlab Harvard Apparatus). Briefly, animals were subjected to one pretraining, six acquisition, one probe, one cue, and three reversal (platform position is changed 180°) sessions. In every session, mice entered the pool from four different positions and were allowed to search the platform for 60 s. For each session, the latency to reach the platform, the total distance swum, the time spent in periphery, the swimming speed, and the time spent floating were analyzed. The average of the four entry points was used as the final measure. In the removal session, the time spent in each quadrant was used as a measure of visuospatial memory.

Novel object recognition test

Mice were placed in a 70 × 70 cm^2 open field arena (Panlab Harvard Apparatus), and their behavior was registered. On day 1, behavior of the mice was registered for 5 min, and the following parameters were analyzed: distance traveled, resting time, and time spent in the center of the arena versus the total arena. Ninety minutes later, mice were put back into the arena with one central object for 5 min (habituation phase). On the following day, two identical objects were placed in the arena, and the time spent exploring each object was registered for 10 min (familiarization phase). Ninety minutes later, one of the objects of the familiarization phase was changed to a new one, and mice were tested for novel object recognition by measuring the time spent exploring each of the objects for 5 min. The discrimination index was calculated as the time spent exploring the new object minus the time spent exploring the familiar object divided by the total time of exploration.

Peripheral ifenprodil and tiagabine administration

Animals were submitted to the pure contextual fear-conditioning paradigm (see above) and immediately after training received an intraperitoneal injection of either ifenprodil, a NMDA receptor 2B (NR2B) antagonist (1 mg/kg, Sigma) or tiagabine, a GABA reuptake inhibitor (10 mg/kg, Sigma). Twenty-four hours after drug administration, mice were tested for contextual fear memory (as described above).

Stereotaxic surgery

Stereotaxic injections were performed in 2- to 3-month-old mice. Briefly, animals were anesthetized with a mixture of medetomidine (1 mg/kg, i.p.) and ketamine (75 mg/kg, i.p.), and analgesia was provided by buprenorphine injection (0.05 mg/kg, s.c.). After complete loss of reflexes, the head was fixed in a stereotaxic apparatus, and, following mouse brain atlas coordinates (Paxinos and Franklin, 2001), two holes in the skull were opened, using a driller, corresponding to the ventral hippocampus, as follows: anteroposterior, −3.0 mm; mediolateral, ±3.0 mm; dorsoventral, −2.3 mm. Cannulae (outer diameter, 0.5 mm; inner diameter, 0.25 mm; AISI 304 Unimed) were implanted bilaterally and fixed with dental cement. At the end of the surgical procedure, anesthesia was reversed with atipamezole injection (2 mg/kg, s.c.).

Intraventral hippocampus tiagabine administration

After 7 d of recovery from surgery, mice were trained in the pure contextual fear-conditioning paradigm (see above) and immediately after training were injected with tiagabine (1 μg/side from a 2 mg/ml solution; Sigma). The injection was performed in immobilized animals through an internal micro-cannula (outer diameter, 0.2 mm; inner diameter, 0.09 mm; AISI 316L, Unimed), connected with a 15-cm-long polyvinyl chlo-

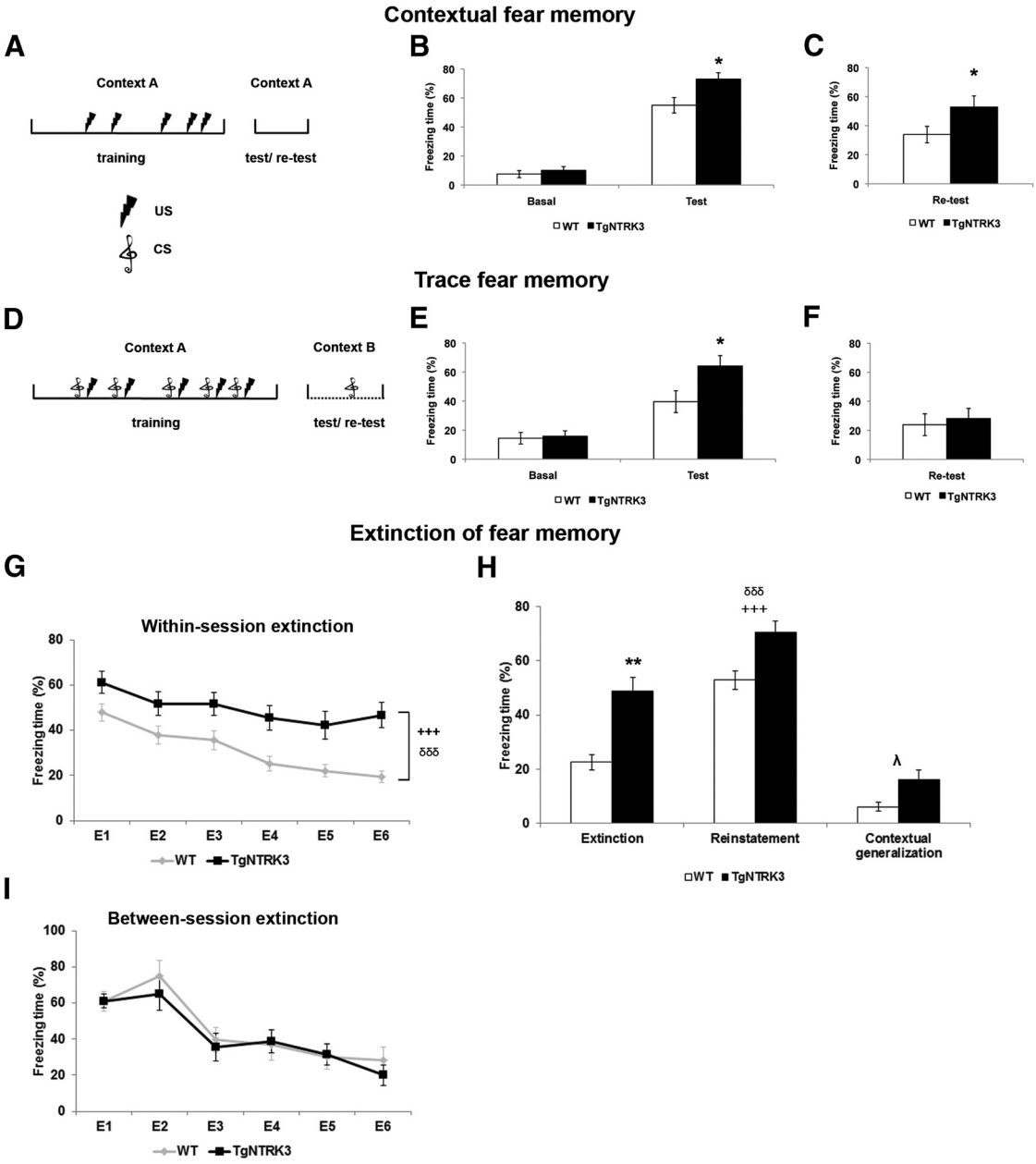

Figure 1. Increased hippocampus-dependent fear memory and resistance to extinction in Tg*NTRK3* mouse model of panic disorder. ***A***, Schematic representation of pure contextual fear-conditioning paradigm. ***B***, ***C***, Twenty-four hours (test) and 1 week (retest) after fear-conditioning training, contextual fear memory is higher in Tg*NTRK3* compared with WT mice (Student's *t* test; $n = 19$ and $n = 22$, respectively). ***D***, Schematic representation of trace fear-conditioning paradigm. ***E***, ***F***, Twenty-four hours, but not 1 week, after trace fear-conditioning training, trace fear memory is higher in Tg*NTRK3* compared with WT mice (Student's *t* test; $n = 21$ and $n = 19$, respectively). ***G***, ***I***, After pure contextual fear conditioning, mice were submitted to different fear extinction paradigms. When tested in the within-session fear extinction paradigm, Tg*NTRK3* mice showed impairment in extinction acquisition (repeated-measures two-way ANOVA; WT, $n = 26$; Tg*NTRK3*, $n = 22$; ***G***) and extinction memory (Student's *t* test; WT, $n = 21$; Tg*NTRK3*, $n = 19$; ***H***) compared with WT. Presentation of the US after extinction resulted in reinstatement of fear both in WT and Tg*NTRK3* mice ($n = 16$ and $n = 14$, respectively), but at higher levels in the transgenic animals (two-way ANOVA, reinstatement vs extinction). This is not due to a generalization of fear since when tested in a new context, freezing time is reduced in both genotypes (WT, $n = 16$; Tg*NTRK3*, $n = 14$) compared with extinction freezing levels (two-way ANOVA, contextual generalization vs extinction). ***I***, In the between-session fear extinction paradigm, no differences in fear extinction were found between genotypes (repeated-measures two-way ANOVA; WT, $n = 13$; Tg*NTRK3*, $n = 14$). Student's *t* test: $^{*}p < 0.05$; $^{**}p < 0.01$; Trial/day/test effect: $^{+++}p < 0.001$. Genotype effect: $^{\delta\delta\delta}p < 0.001$. Genotype × test interaction: $^{\lambda}p = 0.083$.

ride tube (inner diameter, 0.25 mm; F117952, Gilson) to a 5 μl Hamilton syringe. The internal cannula exited 1 mm from the implanted cannula, and the injection was performed with a rate of 250 nl/min. After the injection, animals were placed in their home cage, and 24 h later they were tested for contextual fear memory.

Protein extraction

Mice were anesthetized (mixture of 1.0 mg/kg medetomidine and 0.1 mg/kg ketamine) and intracardially perfused with PBS, pH 7.6. Brains were removed, and hippocampi were dissected and quickly frozen in dry ice. Tissue was homogenized with a 20 gauge needle in 100 μl of lysis buffer (137 mM NaCl, 20 mM Tris-HCl, pH 8.0, 1% NP40, and 10% glycerol) with protease (1 complete pill and 1 mM phenylmethylsulfonyl fluoride) and phosphatase (1 mM NaF and 1 mM Na_3VO_4) inhibitors. After homogenization, samples were vortexed and kept in ice for 3× 5 min. Finally, samples were centrifuged for 30 min, at 13,200 rpm, at 4°C, and the supernatant was collected and stored at −80°C. Protein quantification was performed using the BCA protein assay kit (Thermo Scientific).

Western blot of glutamic acid decarboxylase 65/67 and vesicular glutamate transporter

Fifty micrograms of protein were loaded in 12% polyacrylamide gel, separated by electrophoresis and transferred to nitrocellulose membrane. After blocking (5% milk) membranes were incubated overnight at 4°C with the respective primary antibodies in 0.1% Tris-buffered saline/2.5% milk: rabbit anti-glutamic acid decarboxylase 65/67 (GAD65/67; 1:8000; Millipore), mouse anti-vesicular glutamate transporter 1 (VGLUT1; 1:1000; Synaptic Systems), and rabbit anti-ACTIN (1:5000; Sigma). Adequate secondary antibodies polyclonal goat anti-rabbit Ig/horseradish peroxidase (HRP) and polyclonal rabbit anti-mouse Ig/HRP (Dako) were used. Detection was performed with enhanced chemiluminescence Western blotting detection reagents (GE Healthcare) developed with the Fujifilm LAS 3000 imaging system (R&D Systems), and bands were quantified (MultiGauge version 3.0 software).

Immunofluorescence

Animals were anesthetized (mixture of 0.1 mg/kg ketamine and 1 mg/kg medetomidine) and intracardially perfused with PBS (0.01 M), pH 7.6, followed by 4% paraformaldehyde (PFA). Brains were removed and kept at 4°C in 4% PFA for 24 h for postfixation and then were transferred to a solution of 30% sucrose in PBS for 2 d. A series of coronal sections (40 μm) was obtained using a vibratome (VT1000S; Leica Microsystems) and stored at −20°C in a cryoprotector (30% ethylenoglycol, 30% glycerol, 40% PBS).

Free-floating brain sections were permeabilized with 0.3% Triton X-100 in PBS for 30 min at room temperature (RT) and blocked with 3% bovine serum albumin/0.3% Triton X-100 in PBS for 1 h at RT. Subsequently, sections were incubated overnight at 4°C with the following primary antibodies: mouse anti-VGLUT1 monoclonal antibody (1:200; clone 317G6, Synaptic Systems); guinea pig anti-vesicular GABA transporter (VGAT) polyclonal antibody (1:200; cytoplasmatic domain, Synaptic Systems); rabbit anti-postsynaptic density 95 (PSD-95) polyclonal antibody (1:250; Abcam); and rabbit anti-c-Fos polyclonal antibody (1:500; H-125, Santa Cruz Biotechnology). The following secondary antibodies were used: Alexa Fluor 488 goat anti-mouse IgG, Alexa Fluor 555 goat anti-guinea pig IgG, and Alexa Fluor 594 goat anti-rabbit IgG (1:1000, Life Technologies) for 1 h at RT, protected from light. Finally, sections were mounted and nuclei were stained on glass slides with Vectashield with DAPI (Vector Laboratories).

VGLUT1/VGAT puncta analysis. Pictures were captured in the stratum radiatum (srad) of cornu ammonis 1 (CA1) and CA3 hippocampal subfields using a confocal microscope with a 63× objective and 5× magnification (TCS SPE, Leica Microsystems). For each region, all pictures were captured with identical confocal settings for laser power, gain, and offset levels. Images were imported into IMAGEJ 1.42l (Macbiophotonics), and in each image the negative control background intensity was subtracted for each channel, and converted into binary data and threshold to outline immunopositive puncta. The number and size of VGLUT1 and VGAT puncta per field were quantified using the "analyze particle" function of the software.

VGLUT1/PSD-95 puncta colocalization. Image acquisition and puncta quantification were obtained as described above. For colocalization analysis, threshold images of each channel were superimposed using the Colocalization Highlighter plugin of IMAGEJ 1.42l software, and quantified with the analyze particle function. The average number of VGLUT1/PSD-95 double-positive puncta per genotype was reported normalized for WT values.

Thy1-YFP/c-Fos analysis. Analysis of Thy1-YFP/c-Fos double-positive neurons was performed in the hippocampus from each animal. Images were captured using a confocal microscope (TCS SP5, Leica Microsystems) at 40× magnification. For each area of interest, a 10 μm *z*-stack was captured including the two consecutive pictures in the central part of the CA1 stratum pyramidale (CA1py) and one in the central part of CA3py of the hippocampus. A *z*-maximum projection of each stack was performed by IMAGEJ 1.42l software, the area of interest was drawn, and the number of double-positive neurons was quantified manually. The mean number of positive neurons per area is reported.

c-Fos immunohistochemistry

Brain sections were incubated with 10% MeOH, 3% H_2O_2 in 0.01 M PBS, pH 7.6, for 30 min at RT to block endogenous peroxides and was permeabilized with 0.2% Triton X-100 in PBS (2× 5 min) at RT. Unspecific binding was blocked with 10% fetal bovine serum and 0.2% Triton X-100 in PBS for 1 h at RT. Subsequently, sections were incubated overnight at 4°C with rabbit anti-c-Fos polyclonal primary antibody (1:1000; H-125, Santa Cruz Biotechnology) followed by incubation with biotinylated anti-rabbit secondary antibody (1:300; Vector Laboratories) for 1 h at RT. The staining was developed using the avidin–biotin–peroxidase complex (Vectastain ABC kit, Vector Laboratories) and 3,3-diaminobenzidine as a chromogen. c-Fos-positive neurons were counted manually using an optical microscope (BX51, Olympus) and a CAST grid stereology system (Olympus) in the medial prefrontal cortex [mPFC; prelimbic (PL) and infralimbic (IL) areas], in the CA1py and CA3py layers of hippocampus, and in different nuclei of the amygdala (central, basal, lateral, and the intercalated cell clusters). The mean number of c-Fos-positive neurons per area is reported.

Statistical analysis

A Student's *t* test for independent samples was used to compare data when two independent groups were considered. Two-way ANOVA was used to compare data when two factors were considered (Bonferroni *post hoc* test). Repeated-measures two-way ANOVA was used to compare data when two factors were considered and repeated measures were available (Bonferroni *post hoc*). Statistical significance was set at $p \leq 0.05$, and the results are expressed as the mean ± SEM.

Results

Enhanced hippocampus-dependent fear memories in the Tg*NTRK3* mouse model

In a pure contextual fear-conditioning paradigm (Fig. 1*A*), no differences were found between genotypes during the training session (data not shown). However, in the test session Tg*NTRK3* mice showed a significantly higher freezing time compared with WT mice (Fig. 1*B*; $t_{(39)} = -2.6$, $p = 0.014$). This increased fear response was maintained in the retest session (Fig. 1*C*; $t_{(34.4)} = -2.0$, $p = 0.055$).

In the trace fear-conditioning paradigm (Fig. 1*D*), again Tg*NTRK3* and WT mice learned the CS–US association equally during the training session (data not shown). Strikingly, in the trace fear-conditioning test session, hippocampus-dependent Tg*NTRK3* mice also showed higher freezing time than WT mice (Bangasser et al., 2006; Fig. 1*E*; $t_{(38)} = -2.4$, $p = 0.023$). Both in the retest session of trace fear conditioning (Fig. 1*F*; $t_{(38)} = -0.4$, $p = 0.680$) and in the delay fear-conditioning paradigm (data not shown), two tasks that are less hippocampus dependent (Bangasser et al., 2006; Quinn et al., 2008), no differences were found. These data point to a critical involvement of the hippocampus in the performance of Tg*NTRK3* mice in fear paradigms.

Next, pure contextual fear-conditioned Tg*NTRK3* and WT animals were subjected to different fear extinction paradigms. In the within-session extinction paradigm, Tg*NTRK3* mice showed impaired extinction of acquired fear (Fig. 1*G*; genotype effect: $F_{(1,27)} = 23.2$, $p = 5.0\text{E-}05$; trial effect: $F_{(5,135)} = 11.2$, $p = 5.2\text{E-}09$). In the test session, Tg*NTRK3* mice showed significantly higher freezing behavior compared with WT mice, suggesting that Tg*NTRK3* mice have impaired extinction memory (Fig. 1*H*; $t_{(27.4)} = -3.7$; $p = 0.001$). In the reinstatement session, both genotypes increased the percentage of time spent freezing compared with the extinction session (Fig. 1*H*; test effect: $F_{(1,66)} = 25.5$, $p = 3.8\text{E-}06$) with higher freezing response in Tg*NTRK3* mice (genotype effect: $F_{(1,66)} = 18.1$, $p = 6.9\text{E-}05$). The freezing behavior was not generalized to a new context in either genotype (Fig. 1*H*; genotype × test effect: $F_{(1,66)} = 3.1$; $p = 0.083$). *Post hoc*

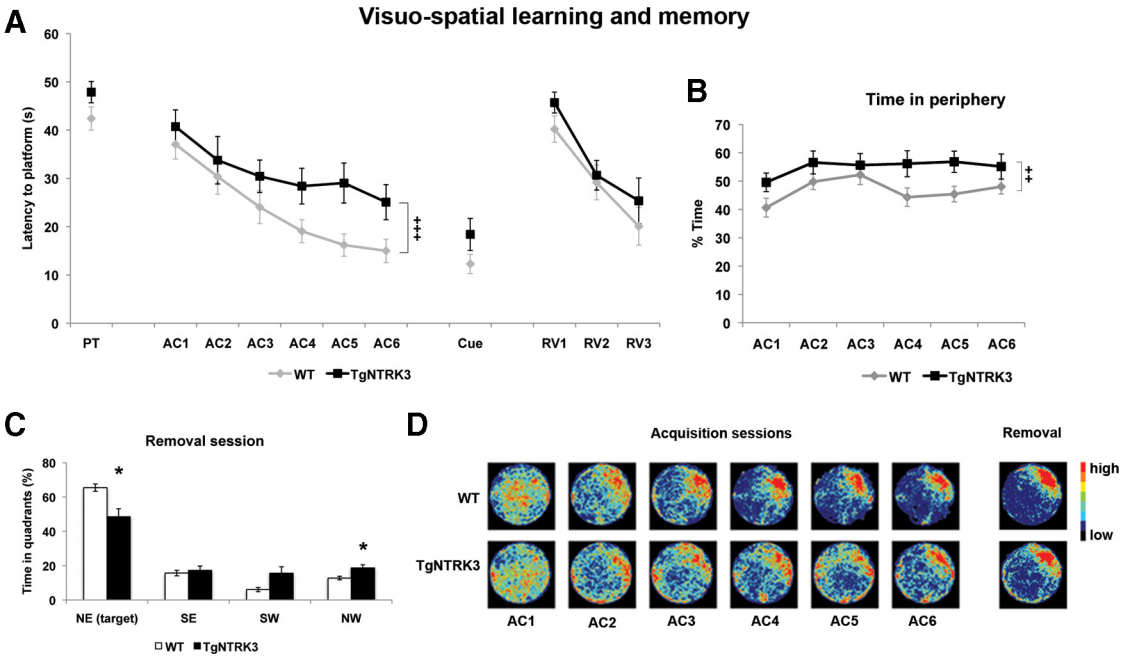

Figure 2. Impaired visuospatial learning and memory of Tg*NTRK3* mice in a water maze paradigm. ***A***, Escape latency of WT and Tg*NTRK3* mice ($n = 18$ and $n = 19$, respectively) was similar in the pretraining and cue (Student's *t* test) and reversal (repeated-measures two-way ANOVA) sessions. During the acquisition sessions, although both genotypes learned the platform position, Tg*NTRK3* mice showed worse performance (repeated-measures two-way ANOVA). ***B***, TgN*TRK3* mice showed a higher percentage of time spent swimming in periphery (repeated-measures two-way ANOVA) during the acquisition sessions. ***C***, In the removal session, WT and Tg*NTRK3* mice showed preference for the target quadrant (repeated-measures two-way ANOVA), but Tg*NTRK3* mice showed a less focused searching strategy, with increased an percentage of time spent swimming in the adjacent quadrants. ***D***, Color-coded map representing the spatial distribution of WT and Tg*NTRK3* mice activity in the acquisition and removal sessions. PT, pretraining; AC1-AC6, acquisition sessions 1–6; RV1-RV3, reversal sessions 1–3; NE, northeast; SE, southeast; SW, southwest; NW, northwest. *Post hoc* comparisons: $^{*}p < 0.05$. Day effect: $^{+++}p < 0.001$, $^{++}p < 0.01$.

Bonferroni comparisons showed no significant differences between genotypes in contextual generalization as opposed to extinction (contextual generalization: WT vs Tg*NTRK3*, $p = 0.15$; extinction: WT vs Tg*NTRK3*, $p = 4.4\text{E-}05$). In the between-session extinction paradigm, analysis of the extinction curves revealed a day effect with no differences between genotypes (Fig. 1*I*; day effect: $F_{(5,60)} = 18.7$, $p = 3.9\text{E-}11$; genotype effect: $F_{(1,12)} = 0.1$, $p = 0.786$). These results suggest that the schedule used for the extinction of fear memory defines its efficiency in Tg*NTRK3* mice.

Impaired spatial memory in the Tg*NTRK3* mice

In the water maze paradigm, Tg*NTRK3* mice showed learning and memory impairments (Fig. 2*A–D*). In the pretraining session, Tg*NTRK3* mice showed a slower swimming speed (Tg*NTRK3* mice, 21.8 cm/s; WT mice, 24.0 cm/s; $t_{(41)} = 2.5$, $p = 0.017$), which did not affect the latency (Fig. 2*A*; $t_{(41)} = -1.7$; $p = 0.093$) or the distance swum (data not shown) to reach the platform, suggesting that motor impairments would not interfere with the visuospatial learning.

No differences for genotype or session were found in the swimming speed or floating behavior along the acquisition, removal, or cue sessions (data not shown). Analysis of the acquisition curves revealed a statistically significant effect of day (Fig. 2*A*,*D*; $F_{(5,100)} = 22.1$; $p = 4.0\text{E-}09$) and a trend toward a main effect for genotype ($F_{(1,20)} = 3.3$, $p = 0.084$) on the escape latencies, indicating that Tg*NTRK3* and WT mice were able to learn but to different levels. The same pattern was found when the distance swum was analyzed (data not shown). This slight learning impairment may in part be due to the thigmotaxic searching strategy used by Tg*NTRK3* mice (Fig. 2*B*,*D*; session effect: $F_{(5,100)} = 3.7$, $p = 0.004$; genotype effect: $F_{(1,20)} = 3.5$, $p = 0.075$).

In the removal session, a significant genotype × quadrant interaction was found (Fig. 2*C*,*D*; $F_{(3,60)} = 5.8$; $p = 0.012$). Both genotypes showed preference for the target quadrant (*post hoc* Bonferroni correction, $p < 0.001$), but Tg*NTRK3* mice showed a less focused searching strategy, spending less time in the target ($p = 0.012$) and more time in the adjacent quadrant ($p = 0.008$) than WT mice. In the cue session, no differences were found between genotypes in the latency to reach the cued platform (Fig. 2*A*) or the percentage of time spent in periphery (data not shown). Analysis of the reversal learning session revealed a significant session effect in the latency to reach the platform (Fig. 2*A*; $F_{(2,32)} = 30.0$; $p = 1.3\text{E-}05$) and distance swum (data not shown), indicating that both genotypes learned the new position of the platform. As in the acquisition, Tg*NTRK3* mice showed a more thigmotaxic behavior (data not shown). Tg*NTRK3* mice swam at a lower speed than WT mice (WT, 24.4 cm/s; Tg*NTRK3*, 19.1 cm/s; $F_{(1,16)} = 5.4$, $p = 0.034$) with no differences in floating behavior (data not shown).

Overall, the impaired performance in the probe session (removal) and a trend toward impairment in the acquisition sessions by Tg*NTRK3* mice suggest that memory cognitive domain is mainly affected rather than learning, as was found for the fear-conditioning paradigms.

Novelty recognition is not altered in the Tg*NTRK3* mice

No differences were found between genotypes during free exploration of the open field (data not shown), in accordance with our previous report (Dierssen et al., 2006), or in the exploration time for the central object during the habituation session (data not shown). In the familiarization phase, WT and Tg*NTRK3* mice equally explored the two objects (Fig. 3*A*; object effect: $F_{(1,17)} = 2.4$, $p = 0.14$; genotype effect: $F_{(1,17)} = 1.1$, $p = 0.31$), and in the

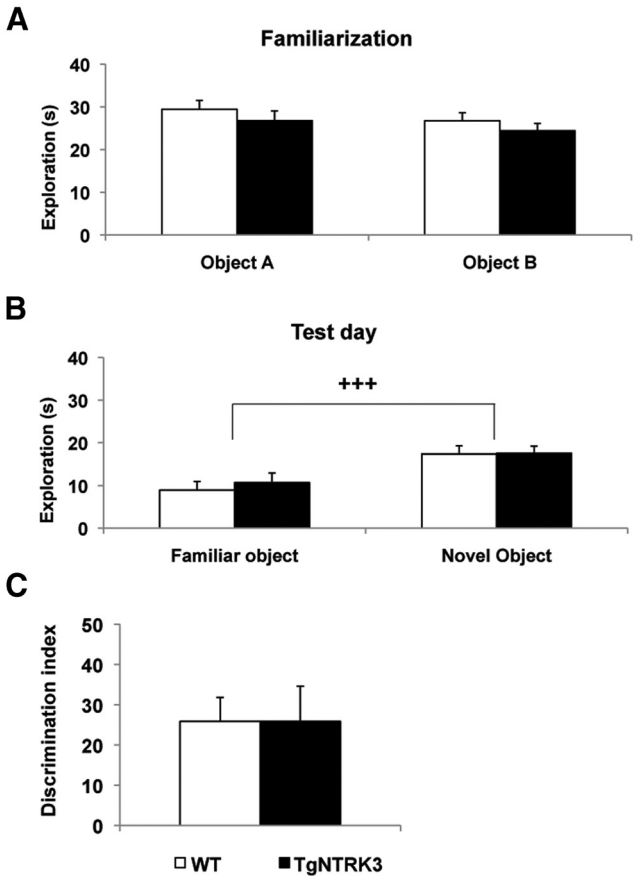

Figure 3. Intact novelty recognition in Tg*NTRK3* mice. ***A***, In the familiarization session, WT and Tg*NTRK3* mice ($n = 18$ and $n = 19$, respectively) showed equal levels of exploration of both objects (repeated-measures two-way ANOVA). ***B***, ***C***, In the test session, both genotypes spent more time exploring the novel object compared with the familiar object (repeated-measures two-way ANOVA) with a similar discrimination index. Novel object effect: $^{+++}p < 0.001$.

test session WT and Tg*NTRK3* mice spent equally more time exploring the novel object (Fig. 3*B*,*C*; object effect: $F_{(1,17)} = 23.9$, $p = 0.0001$; genotype effect: $F_{(1,17)} = 1.1$, $p = 0.30$).

Tg*NTRK3* mice show a differential neuronal activation pattern in naive, fear-conditioned, and fear extinction conditions

To analyze how the fear circuit is activated in Tg*NTRK3* compared with WT mice, we quantified the number of c-Fos-positive cells in the hippocampus (specifically in the CA1py and CA3py subfields, where cell bodies of pyramidal neurons are located), in several amygdala nuclei (basolateral, central and intercalated cell clusters), and in mPFC (focusing on prelimbic and infralimbic regions that modulate fear responses) brain regions upon pure contextual fear conditioning and extinction compared with the naive state.

Neuronal activation pattern in naive and fear-conditioned mice

In the hippocampus, we quantified the number of c-Fos-positive cells.

One hour after fear-conditioning training, we observed increased activation of both dorsal (data not shown) and ventral hippocampus CA1py and CA3py layers compared with the naive condition in both genotypes [Fig. 4*A*,*B*; fear-conditioning (FC) training effect: ventral CA1py: $F_{(1,18)} = 25.6$, $p = 8.2\text{E-}05$; CA3py: $F_{(1,18)} = 28.9$, $p = 4.2\text{ E-}05$]. However, the ventral hippocampus of Tg*NTRK3* mice showed a significant genotype × FC interaction in ventral CA1py layer ($F_{(1,18)} = 4.5$, $p = 0.048$). In this subfield, fear conditioning activates at much higher levels in Tg*NTRK3* neurons (WT-naive vs WT-FC, $p = 0.052$; Tg*NTRK3*-naive vs Tg*NTRK3*-FC, $p = 7.9\text{E-}05$; Tg*NTRK3*-FC vs WT-FC, $p = 0.013$). Also, a higher number of c-Fos-positive cells in CA3py layer of Tg*NTRK3* mice compared with WT mice was found (genotype effect: $F_{(1,18)} = 6.1$, $p = 0.024$). The increased activation of ventral CA1py and CA3py layers in Tg*NTRK3* mice could contribute to the increased contextual fear memory observed in the transgenic animals.

We also analyzed the activation of the amygdala, the core brain region in the fear circuit. In the centrolateral nucleus of amygdala (CeL), the number of c-Fos-positive cells in both WT and Tg*NTRK3* mice was reduced after fear conditioning (Fig. 4*A*,*C*; FC effect: $F_{(1,17)} = 9.7$, $p = 0.006$). In the centromedial nucleus of amygdala (CeM), which sends projections to regions that finally orchestrate the fear response, a significant genotype × FC interaction was detected (Fig. 4*A*,*C*; $F_{(1,17)} = 5.3$; $p = 0.034$), and *post hoc* comparisons revealed that in naive conditions the CeM of Tg*NTRK3* mice was more activated than in WT mice (WT-naive vs Tg*NTRK3*-naïve, $p = 0.003$). After fear conditioning, neuronal activation was significantly increased in Tg*NTRK3* (Tg*NTRK3*-naive vs Tg*NTRK3*-FC, $p = 4.0\text{ E-}04$) but not in WT mice (WT-naive vs WT-FC, $p = 0.329$), with a significant genotype difference (WT-FC vs Tg*NTRK3*-FC, $p = 2.2\text{ E-}06$). In addition, the ventral hippocampal and mPFC inputs mostly impinge on basolateral amygdala (BLA) and intercalated nuclei (ITC) to regulate fear responses. We did not find any differences in the number of c-Fos-positive cells in the lateral (LA) and basal (BA) subdivisions of the BLA between genotypes, both in naive and fear-conditioning states (Fig. 4*C*; LA: genotype effect, $F_{(1,17)} = 0.81$, $p = 0.38$; FC effect, $F_{(1,17)} = 0.99$, $p = 0.34$; genotype × FC interaction, $F_{(1,17)} = 0.66$, $p = 0.43$; BA: genotype effect, $F_{(1,17)} = 1.53$, $p = 0.23$; FC effect, $F_{(1,17)} = 0.09$, $p = 0.76$; genotype × FC interaction, $F_{(1,17)} = 0.61$, $p = 0.44$). Interestingly, in the lateral ITC (lITC) naive Tg*NTRK3* animals showed an increased number of c-Fos-positive neurons compared with their respective WT group, and fear conditioning reduced the number of activated cells only in Tg*NTRK3* animals (Fig. 4*C*; genotype × FC interaction: $F_{(1,17)} = 6.0$, $p = 0.025$; Bonferroni *post hoc* test: WT-naive vs Tg*NTRK3*-naïve, $p = 0.023$; Tg*NTRK3*-naive vs Tg*NTRK3*-FC, $p = 0.039$). In the medial ITC (mITC), we observed that upon fear conditioning a differential activation pattern is produced in WT and Tg*NTRK3* mice. Fear conditioning increased the number of c-Fos-positive cells in WT but not in Tg*NTRK3* mice (Fig. 4*C*; genotype × FC interaction: $F_{(1,17)} = 7.4$, $p = 0.014$; Bonferroni *post hoc* test: WT-naive vs WT-FC, $p = 0.049$; Tg*NTRK3*-naive vs Tg*NTRK3*-FC, $p = 0.10$; WT-FC vs Tg*NTRK3*-FC, $p = 0.028$).

In the mPFC, which is thought to play an important role in inhibiting the amygdala and blocking fear responses, fear conditioning did not change the number of activated neurons in either WT or Tg*NTRK3* mice compared with the naive condition in the PL area (Fig. 4*A*,*D*; $F_{(1,18)} = 3.4$; $p = 0.081$). However, in the IL region a significant genotype × FC effect is revealed (Fig. 4*A*,*D*; $F_{(1,18)} = 4.5$, $p = 0.048$), and *post hoc* comparisons showed that fear conditioning reduced neuronal activation in Tg*NTRK3* but not in WT mice (Tg*NTRK3*-naive vs Tg*NTRK3*-FC, $p = 1.2\text{E-}04$; WT-naive vs WT-FC, $p = 0.08$).

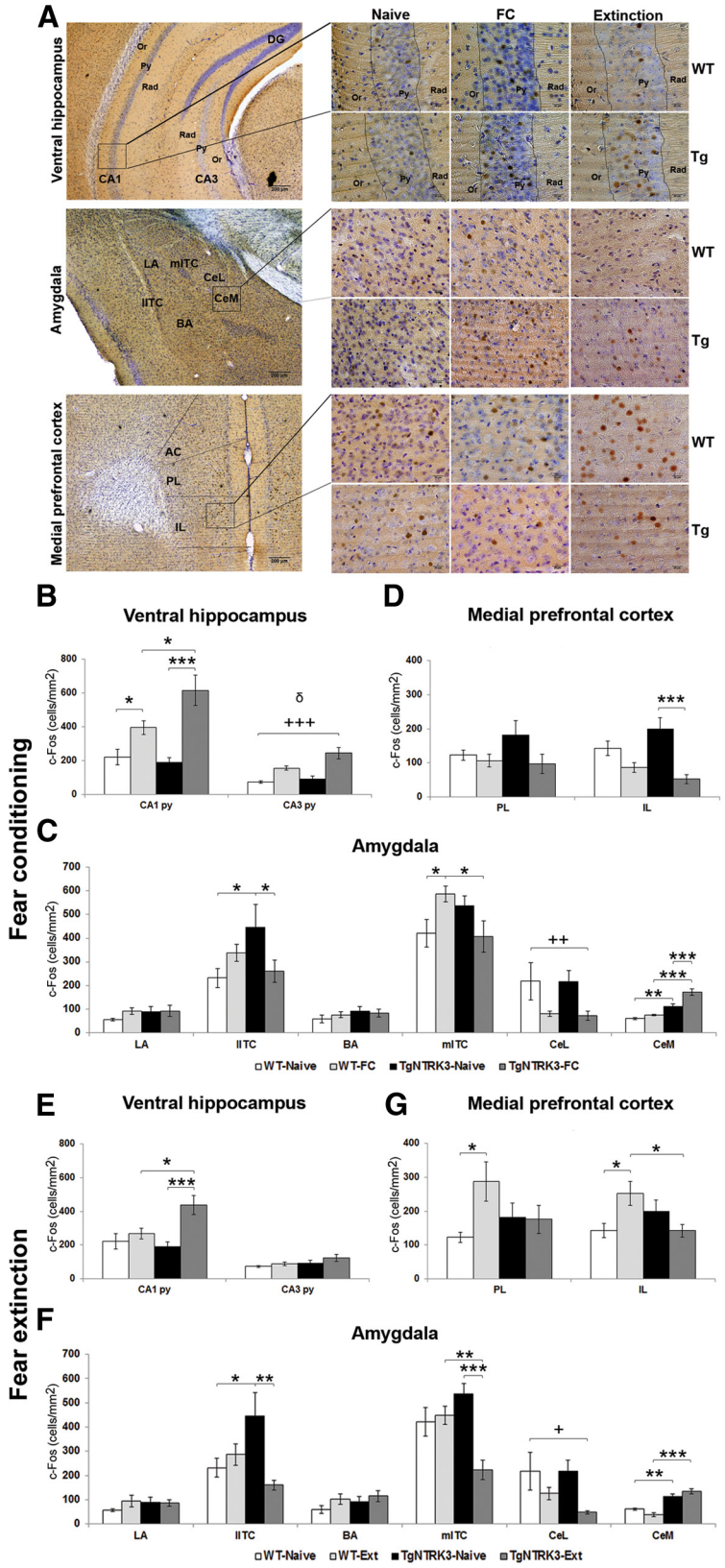

Figure 4. Altered neuronal activation pattern of the Tg*NTRK3* amygdala–hippocampus–mPFC fear circuit in naive, fear-conditioned and fear-extinguished states. ***A***, Representative photomicrographs of c-Fos immunohistochemistry of WT and Tg*NTRK3* ventral hippocampus, amygdale, and mPFC brain regions in naive, FC, and Ext states. ***B–D***, Number of c-Fos-positive cells in naive ($n = 5$ per genotype) and FC training groups ($n = 6$ per genotype) in ventral hippocampus (***B***), amygdala (***C***), and mPFC (***D***) brain regions. In brief, a higher neuronal activation was found in ventral hippocampus CA1py and CeM in Tg*NTRK3* compared with WT mice. In contrast, in the lITC and mITC of the amygdala and in the IL region of the mPFC a marked reduction of c-Fos expression is observed after FC in Tg*NTRK3* mice. ***E–G***, Number of c-Fos-positive cells in naive ($n = 5$ per genotype) and Ext groups ($n = 5$ per genotype) in ventral hippocampus (***E***), amygdala (***F***), and mPFC (***G***) brain regions. Briefly, Tg*NTRK3* mice showed higher c-Fos expression in ventral hippocampus CA1py after fear extinction compared with other groups. In the CeM nucleus of the amygdala, the number of c-Fos cells was already higher in naive Tg*NTRK3* compared with WT mice, and fear extinction did not reduce this number in transgenic animals. In both the lITC and the mITC of Tg*NTRK3* mice, fear extinction reduced the number of c-Fos neurons, without any change in WT mice. Moreover, fear extinction activated PL and IL regions in WT but not in Tg*NTRK3* mice. Or, *Stratum oriens*; DG, dentate gyrus; AC, anterior cingulum. Genotype effect: $^{\delta}p < 0.05$. FC/Ext effect: $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$. *Post hoc* comparisons: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. A two-way ANOVA was used for all statistical comparisons.

Neuronal activation after extinction of conditioned fear

In the ventral hippocampus, fear extinction increased CA1py neuronal activation in Tg*NTRK3* mice, but not in WT mice, compared with the naive condition (Fig. 4*A*,*E*; genotype × fear Ext: $F_{(1,16)} = 5.7$, $p = 0.03$; Bonferroni *post hoc* test: Tg*NTRK3*-naive vs Tg*NTRK3*-Ext, $p = 7.1E\text{-}04$; WT-Ext vs Tg*NTRK3*-Ext, $p = 0.012$). No differences were found in ventral CA3py layer (genotype effect: $F_{(1,16)} = 3.2$, $p = 0.094$; Ext effect: $F_{(1,16)} = 2.4$, $p = 0.14$; genotype × Ext interaction: $F_{(1,16)} = 0.22$, $p = 0.65$). Again, no significant differences were observed in the dorsal CA1py and CA3py hippocampal subfields (data not shown).

In the amygdala, 1 h after fear extinction training, a significant Ext effect was found in the CeL nucleus (Fig. 4*A*,*F*; $F_{(1,16)} = 7.5$, $p = 0.015$), with extinction training reducing c-Fos numbers in both genotypes. Quantification of c-Fos-immunopositive nuclei revealed increased numbers of c-Fos-positive cells in the CeM of Tg*NTRK3* compared with WT mice (Fig. 4*A*,*F*; genotype × Ext interaction: $F_{(1,16)} = 6.4$, $p = 0.022$), with a higher number of c-Fos-positive cells detected in Tg*NTRK3* mice both in the naive condition and after the extinction paradigm (WT-naive vs Tg*NTRK3*-naive, $p = 0.001$; WT-Ext vs Tg*NTRK3*-Ext, $p = 1.5E\text{-}06$). In both the LA and BA subdivisions of the BLA nucleus, no differences were found in the number of c-Fos-positive cells between genotypes, in naive and fear extinction states (Fig. 4*F*; LA: genotype effect, $F_{(1,16)} = 0.43$, $p = 0.52$; Ext effect, $F_{(1,16)} = 0.87$ $p = 0.37$; genotype × Ext interaction, $F_{(1,16)} = 1.43$, $p = 0.25$; BA: genotype effect, $F_{(1,16)} = 1.29$, $p = 0.27$; Ext effect, $F_{(1,16)} = 2.48$, $p = 0.14$; genotype × Ext interaction, $F_{(1,16)} = 0.27$, $p = 0.61$). In the lITC, naive Tg*NTRK3* animals presented an increased number of c-Fos-

positive neurons compared with their respective WT group, and fear extinction reduced the number of activated cells in Tg*NTRK3* mice, with no effect in WT animals (Fig. 4*F*; genotype × Ext interaction: $F_{(1,16)} = 8.62$, $p = 0.01$; Bonferroni *post hoc* test: WT-naive vs Tg*NTRK3*-naïve, $p = 0.019$; Tg*NTRK3*-naive vs Tg*NTRK3*-Ext, $p = 0.003$). In the mITC, no differences in the number of c-Fos cells were observed between genotypes in the naive condition. However, fear extinction training, again, reduced the number of activated cells in Tg*NTRK3* but not in WT controls (Fig. 4*F*; genotype × Ext interaction: $F_{(1,16)} = 13.82$, $p = 0.002$; Bonferroni *post hoc* test: WT-naive vs Tg*NTRK3*-naïve, $p = 0.097$; Tg*NTRK3*-naive vs Tg*NTRK3*-Ext, $p = 1.8E\text{-}04$; WT-Ext vs Tg*NTRK3*-Ext, $p = 0.003$). Extinction training activated the mPFC region of WT but not Tg*NTRK3* mice, with a significant genotype × Ext interaction found in both the PL and IL areas (Fig. 4*A*,*G*; PL: $F_{(1,16)} = 4.1$, $p = 0.061$; IL: $F_{(1,16)} = 8.6$, $p = 0.01$). *Post hoc* comparisons revealed that fear extinction increased the number of c-Fos-positive cells in PL region only in WT animals (WT-naive vs WT-Ext, $p = 0.014$). In IL, the number of c-Fos-positive cells was higher in the WT-Ext group than in WT-naive or Tg*NTRK3*-Ext group (WT-naive vs WT-Ext, $p = 0.014$; WT-Ext vs Tg*NTRK3*-Ext, $p = 0.014$).

In summary, fear extinction training does not activate mPFC in Tg*NTRK3* leading to the reduced inhibition of amygdala, as shown by higher neuronal activation of CeM nucleus. The higher neuronal activation observed in the CA1py layer of ventral hippocampus of Tg*NTRK3* mice would contribute to the increased context fear.

To determine the exact nature of the activated cells found in the ventral hippocampus region, c-Fos staining in Thy1-YFP:*NTRK3* double-transgenic mice allowed the visualization of activated pyramidal neurons. Thy1-YFP:*NTRK3* mice have similar numbers of Thy1-YFP cells as Thy1-YFP:WT littermates (data not shown). However, we found a higher number of c-Fos/Thy1-YFP double-positive cells in Thy1-YFP:*NTRK3* mice compared with Thy1-YFP:WT mice in the ventral hippocampus CA1py layer ($t_{(8)} = -2.9$, $p = 0.0186$), but not in CA3py layer ($t_{(8)} = -1.3$, $p = 0.23$), after fear conditioning (Fig. 5*A*,*B*). Thus, the enhanced CA1 activation detected in c-Fos experiments corresponds with a higher number of activated pyramidal excitatory cells in ventral hippocampus CA1py layer of Tg*NTRK3* mice, possibly resulting in higher excitability of this region.

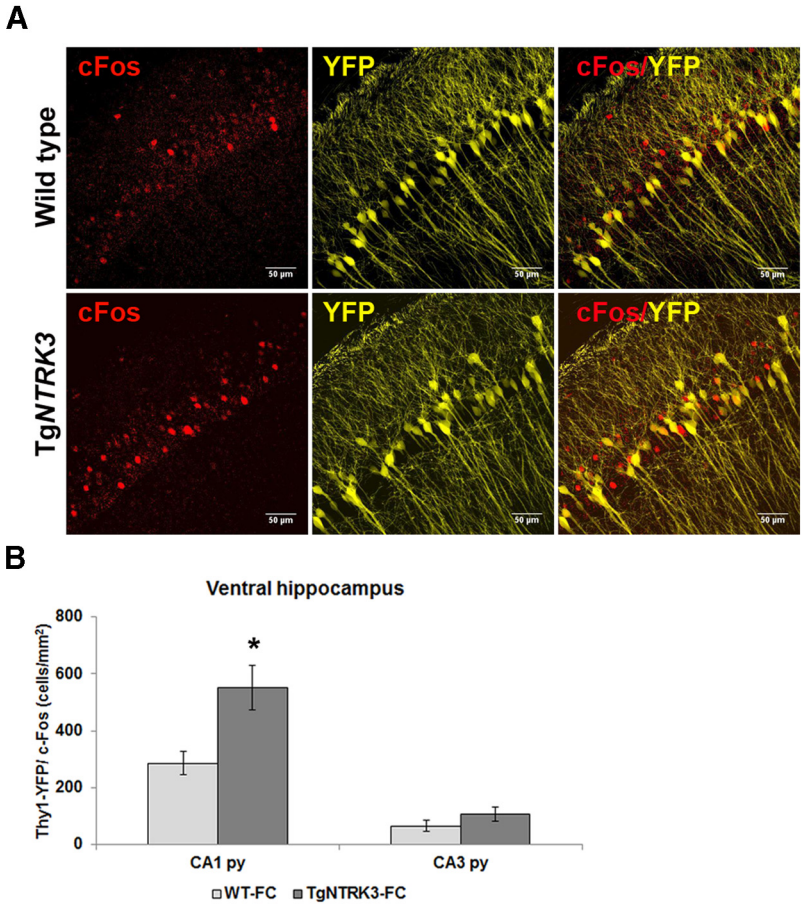

Figure 5. Increased activation of excitatory neurons of ventral hippocampus CA1py layer in Tg*NTRK3* mice, upon fear conditioning. ***A***, Representative photomicrographs of c-Fos immunofluorescence and Thy-1-YFP-positive neurons in ventral hippocampus CA1py region. ***B***, Quantification of c-Fos/YFP double-positive neurons ($n = 5$ per genotype) showed a remarkable increase in the number of activated excitatory cells in ventral hippocampus CA1py layer of Tg*NTRK3* compared with WT animals (Student's *t* test). Student's *t* test: $^*p < 0.05$.

Hippocampal overexcitability is due to an increased glutamatergic load

Western blot analysis in total hippocampus extracts showed higher protein levels of VGLUT1 (Fig. 6*A*; $t_{(8.3)} = -2.9$, $p = 0.018$) and lower levels of GAD65/67 (Fig. 6*B*; $t_{(16)} = 2.3$, $p = 0.038$) in Tg*NTRK3* mice, indicating an overall dysbalance toward excitation.

To further confirm and additionally map the glutamatergic/GABAergic dysbalance, immunofluorescence staining against VGLUT1 and VGAT presynaptic markers was performed and analyzed in the stratum radiatum layers of CA1 and CA3 hippocampal regions, a subfield where pyramidal neurons receive their presynaptic inputs. Double immunofluorescence for VGLUT1 and VGAT (Fig. 6*C*) showed a significant increase in the VGLUT1/VGAT puncta ratio in CA1 srad (Fig. 6*D*; $t_{(14)} = -2.6$, $p = 0.020$), but not in CA3srad ($t_{(14)} = -0.8$, $p = 0.453$) of ventral hippocampus of Tg*NTRK3* mice. No differences were found in the size of the VGLUT1 and VGAT puncta in CA1 and CA3 (data not shown) or in the number of VGLUT1/PSD-95 puncta (excitatory synaptic contacts; Fig. 6*E*; CA1: $t_{(14)} = 0.1$, $p = 0.92$; CA3: $t_{(14)} = 0.06$, $p = 0.95$). Again, differences were detected only in the ventral hippocampus, but not in the dorsal hippocampus (data not shown).

Tiagabine, but not ifenprodil, administration rescues the fear phenotype in Tg*NTRK3* mice

We attempt to rescue the observed hippocampal phenotype by injecting ifenprodil or tiagabine immediately after contextual fear-conditioning training. Peripheral administration of an active ifenprodil dose (inducing hypolocomotion in both genotypes; data not shown) had no effect in contextual fear memory of WT and Tg*NTRK3* mice, at 24 h (Fig. 7*A*; treatment effect:

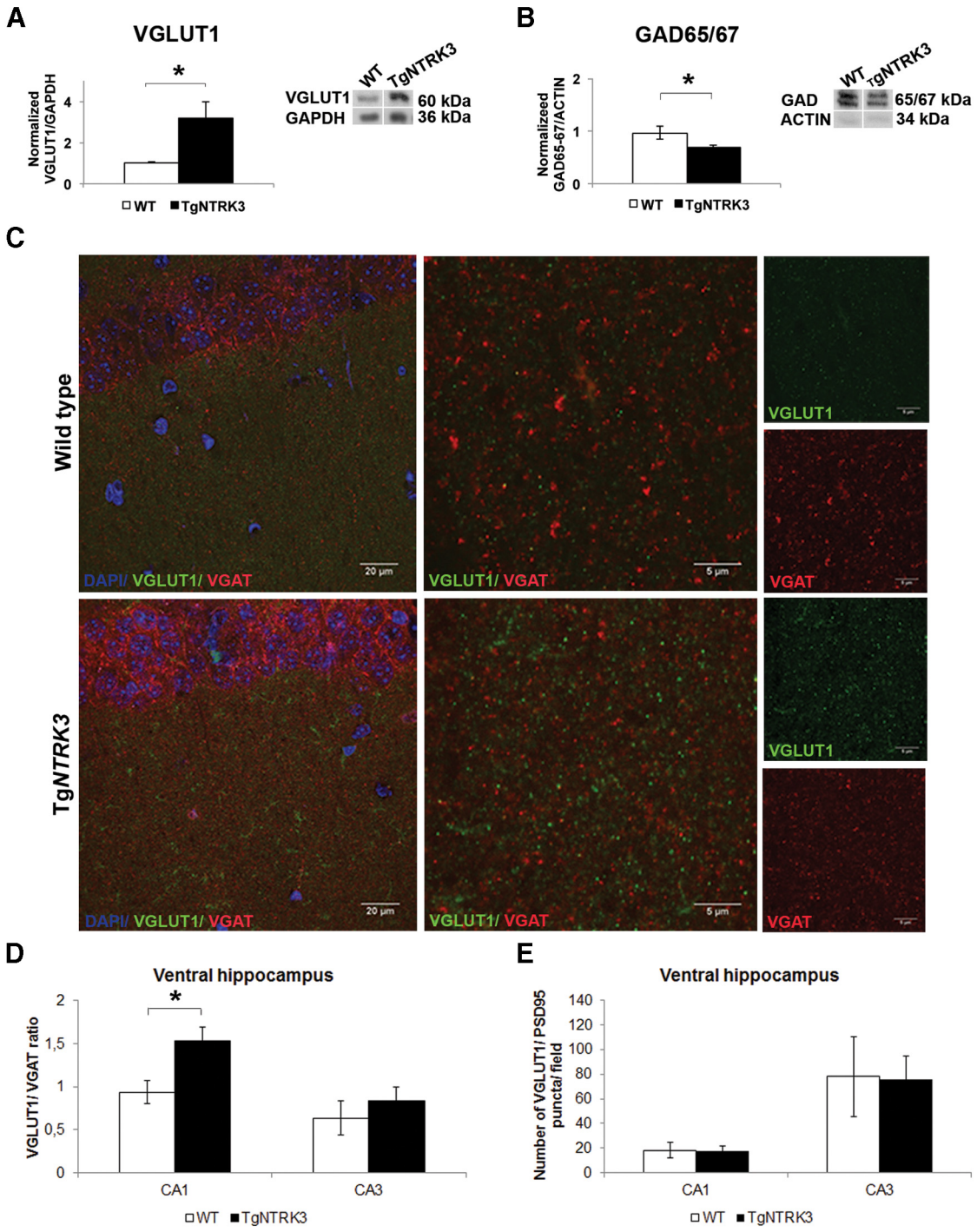

Figure 6. Excitatory-to-inhibitory dysbalance toward hyperexcitability in Tg*NTRK3* mice ventral hippocampus CA1 region. ***A***, ***B***, Western blot analysis of hippocampal extracts showed that expression levels of VGLUT1 (WT, $n = 6$; Tg*NTRK3*, $n = 9$; ***A***) are increased and those of GAD65/67 (WT, $n = 8$; Tg*NTRK3*, $n = 10$; ***B***) are reduced in Tg*NTRK3* compared with WT animals (Student's *t* test). ***C***, Representative photomicrographs of VGLUT1 and VGAT puncta immunofluorescence in ventral hippocampus CA1srad layer in WT and Tg*NTRK3* mice. ***D***, Quantitative analyses of VGLUT1 and VGAT puncta in CA1srad and CA3srad showed a remarkable increase in VGLUT1/VGAT ratio in Tg*NTRK3* CA1srad layer with respect to WT (Student's *t* test: WT, $n = 5$; Tg*NTRK3*, $n = 6$). ***E***, No differences were found between genotypes (Student's *t* test: $n = 8$ per genotype) when the number of excitatory (VGLUT1/PSD-95-positive) contacts were analyzed. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Student's *t* test: $^{*}p < 0.05$.

$F_{(1,36)} = 0.8$, $p = 0.38$; genotype effect: $F_{(1,36)} = 5.7$, $p = 0.023$; genotype × treatment effect: $F_{(1,36)} = 0.03$, $p = 0.87$).

However, peripheral tiagabine (GABA reuptake inhibitor) yielded a significant genotype × treatment interaction (Fig. 7*B*; $F_{(1,30)} = 12.2$, $p = 0.0015$). *Post hoc* comparisons showed that tiagabine decreased contextual fear memory in Tg*NTRK3* mice to WT levels, with no effect in WT mice (Tg*NTRK3*-saline vs Tg*NTRK3*-tiagabine, $p = 6.9\text{E-}06$; WT-saline vs Tg*NTRK3*-saline, $p = 0.010$; WT-tiagabine vs Tg*NTRK3*-tiagabine, $p = 0.036$). Interestingly, when tiagabine was administered locally into the ventral hippocampus through implanted cannulae (Fig. 7*C*), both WT and Tg*NTRK3* mice showed a significant reduc-

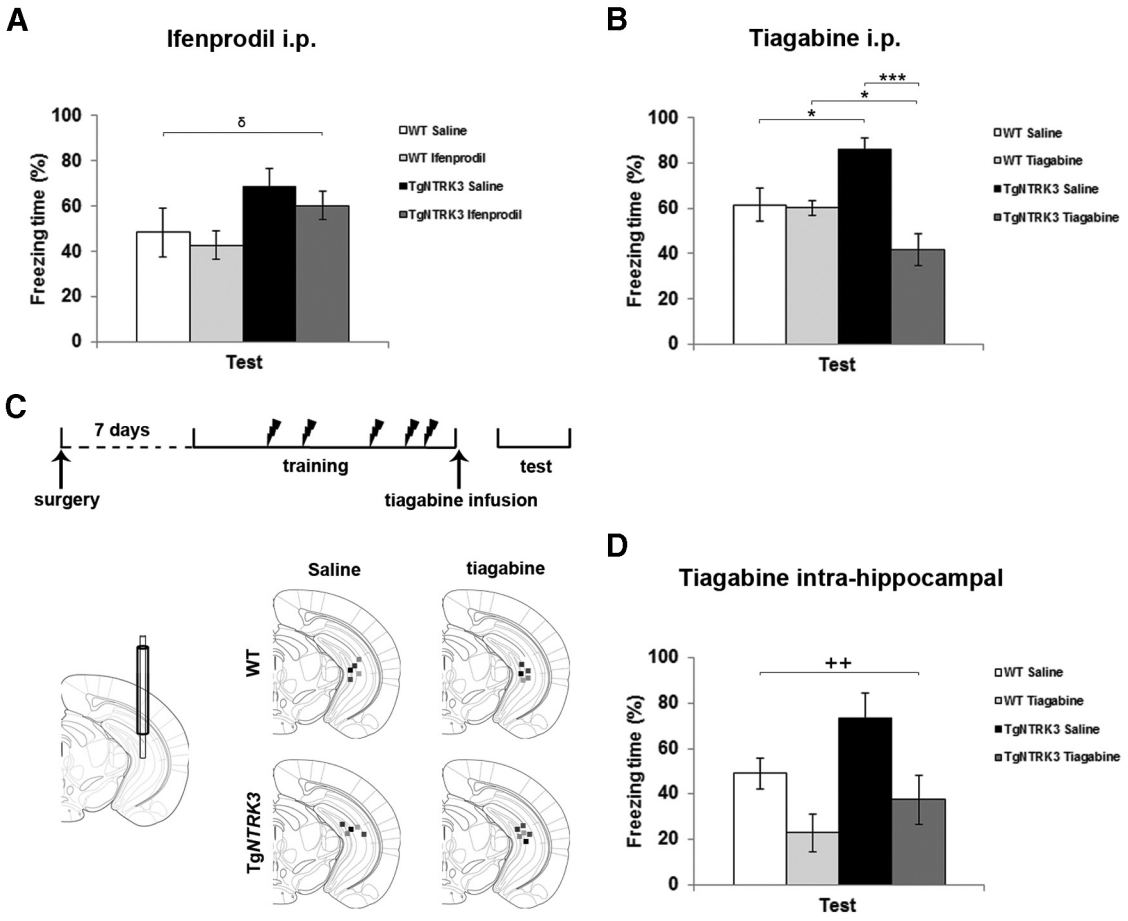

Figure 7. Increased fear memory in Tg*NTRK3* mice is normalized upon localized hippocampus GABA transmission enhancement. ***A***, ***B***, WT and Tg*NTRK3* mice were submitted to the pure contextual FC paradigm and immediately after the training session received intraperitoneal injection with saline, 1 mg/kg ifenprodil or 10 mg/kg tiagabine. Mice were tested for pure contextual fear memory 24 h after drug administration, and data showed that ifenprodil has no effect on fear memory in both WT and Tg*NTRK3* animals (two-way ANOVA: WT-saline, $n = 9$; WT-ifenprodil, $n = 10$; Tg*NTRK3*-saline, $n = 10$; Tg*NTRK3*-ifenprodil, $n = 11$; ***A***) and that tiagabine administration reduced the freezing time of Tg*NTRK3* mice and rescue contextual fear memory to WT levels (***B***), with no effect in WT mice (two-way ANOVA: WT-saline, $n = 7$; WT-tiagabine, $n = 8$; Tg*NTRK3*-saline, $n = 9$; Tg*NTRK3*-tiagabine, $n = 10$). ***C***, Bilateral injection cannulae were implanted by stereotaxic surgery in the ventral hippocampus (following coordinates from Paxinos and Franklin, 2001) of WT and Tg*NTRK3* mice. After recovery from surgery, mice were submitted to the pure contextual fear conditioning and immediately after training infused with saline or 1 μg/side of tiagabine. Schematic brain sections show the injection target site in the ventral hippocampus and the real infusion point in each mouse. ***D***, Twenty-four hours after tiagabine infusion, mice were tested for contextual fear memory. Quantification of freezing time shows that tiagabine infusion reduced fear response in both genotypes, compared with saline-infused groups (two-way ANOVA: WT-saline, $n = 5$; WT-tiagabine, $n = 5$; Tg*NTRK3*-saline, $n = 5$; Tg*NTRK3*-tiagabine, $n = 6$). Genotype effect: $^{\delta}p < 0.05$. Treatment effect: $^{++}p < 0.01$. *Post hoc* comparisons: $^{*}p < 0.05$, $^{***}p < 0.001$.

tion of freezing response at the test phase (Fig. 7*D*; treatment effect: $F_{(1,17)} = 10.3$, $p = 0.005$) and a trend for a significant enhanced fear response in Tg*NTRK3* mice compared with WT (genotype effect: $F_{(1,17)} = 4.0$, $p = 0.061$). Thus, the increased contextual fear memory phenotype observed in Tg*NTRK3* mice is rescued by specifically strengthening GABAergic transmission in the ventral hippocampus, during the consolidation phase of fear memory.

Discussion

We here show an increased and resistant-to-extinction contextual fear memory in Tg*NTRK3* mice, a validated model of PAND. This phenotype can be explained by the abnormal activation of the amygdala–hippocampus–medial prefrontal cortex fear network and sustained hyperexcitability of CA1py layer of ventral hippocampus, as supported by the rescue of contextual fear through administration of tiagabine, a GABA reuptake inhibitor. These results shed light on the mechanisms of the increased conditionability and resistance to extinction observed in PAND patients.

First, we found that training Tg*NTRK3* mice in hippocampal-dependent fear-conditioning paradigms resulted in lastingly higher fear memory compared with WT controls (Fig. 1*A–F*). In addition to the persistence of such enhanced fear memory, deficits in fear extinction are typical for anxiety disorders, such as post-traumatic stress disorder and PAND (American Psychiatric Association, 2000; Blechert et al., 2007; Michael et al., 2007). When submitted to a within-session paradigm, Tg*NTRK3* mice showed impaired fear extinction acquisition and memory (Fig. 1*G*,*H*), but in the between-session paradigm we found no differences between genotypes (Fig. 1*I*). The presence of a consolidation phase after the presentation of each nonpaired CS, as is the case in the between-session paradigm, should contribute to the differences observed between the extinction paradigms. Strikingly, a similar dissociation of within-session and between-session extinction has previously been reported for the mGLUR7 agonist AMN082, which impaired the former, but facilitated retention of the latter (Toth et al., 2012). Activation of mGLUR7 induces phosphorylation changes of MAPK signaling pathways

(Tian et al., 2010), converging with TrkC-mediated signaling. The differences found between the two extinction paradigms may have implications for PAND clinical outcome when considering cognitive behavioral therapy in panic or other anxiety disorders. When Tg*NTRK3* mice were tested for contextual generalization (Fig. 1*H*), although we did not see a statistical significant difference in freezing levels, compared with WT, Tg*NTRK3* mice freeze more. We can speculate that transgenic animals are starting to develop a generalization of contextual fear response, a well described endophenotype in panic disorder that has been related to pattern separation and adult hippocampal neurogenesis (for review, see Kheirbek et al., 2012).

Interestingly, c-Fos expression was induced in pyramidal cells of ventral hippocampal area CA1 of both genotypes after fear conditioning, but to a much higher extent in Tg*NTRK3* animals (Figs. 4*A*,*B*, 5*A*,*B*). Despite this increased hippocampal activity, Tg*NTRK3* mice showed mild impairments in another hippocampus-dependent memory test, the Morris water maze (MWM; Fig. 2), and no apparent deficits in the novel object recognition test (Fig. 3), suggesting a differential and complex effect of TrkC on hippocampal memory function. Fear conditioning and MWM tasks do rely on the hippocampus but are ascribed to different hippocampal subcircuits. While fear conditioning is mainly dependent on the ventral hippocampus, the MWM depends mainly on the dorsal hippocampus (Zhang et al., 2004). In fact, most of the histochemical differences we found were specific to the ventral hippocampus, with the dorsal hippocampus showing only subtle differences or no differences at all (data not shown).

Our results suggest that fear memories in Tg*NTRK3* mice could be modulated by recruiting different neural circuits. We studied the neural activation pattern of the amygdala, the hippocampus, and the mPFC, which are critically involved in fear behavior. Naive Tg*NTRK3* mice showed increased c-Fos labeling in the CeM of the amygdala (Fig. 4*C*,*F*), which orchestrates conditioned autonomic and motor responses (LeDoux et al., 1988) as well as innate fear (Nanda et al., 2008). The basal overactivation of this region may thus explain the increased innate fear response of Tg*NTRK3* mice that was observed previously (Dierssen et al., 2006). Ventral hippocampal and mPFC inputs mostly impinge on LA/BLA and ITC of the amygdala to regulate fear (Sierra-Mercado et al., 2011; Bienvenu et al., 2012). In our study, fear-conditioning and extinction processes resulted in an important hypoactivation of the ITCs in Tg*NTRK3* mice, without affecting LA/BA activation pattern, suggesting that the disrupted fear may result from an impaired processing of incoming information into the ITCs. The fact that no differences are observed at the LA/BLA might suggest the occurrence of compensatory mechanisms at these nuclei. Upon contextual fear-conditioning training, c-Fos expression increased more strongly in CeM amygdala of Tg*NTRK3* mice (Fig. 4*C*), which is in line with their increased freezing response (Fig. 1*B*,*E*). In contrast, in the IL region of mPFC, fear-conditioning training led to an inhibition of c-Fos expression in Tg*NTRK3* but not in WT mice (Fig. 4*D*). These results indicate that fear-related information is processed by distinct spatial patterns of neuronal activity in the fear network in PAND, in which increased activation of ventral hippocampus and inhibition of mPFC during fear conditioning, converge with a hyper-reactive CeM, thus amplifying the fear signal. Importantly, the over-reactivity of both the ventral hippocampus and the CeM were resistant to extinction training in Tg*NTRK3* animals (Fig. 4*E*,*F*). Moreover, the mPFC (PL and IL regions), which is involved in the extinction of conditioned fear (Morgan et al., 1993; Quirk et al., 2000, 2003; Berretta et al., 2005), was not activated upon fear extinction in Tg*NTRK3* mice (Fig. 4*G*). Inactivation of the IL region impairs the acquisition of fear extinction and extinction memory (Milad and Quirk, 2002; Sierra-Mercado et al., 2011), while the hippocampus is required for both fear expression and extinction memory, serving to disambiguate conflicting CS memories and determine performance (Holt and Maren, 1999; Corcoran and Maren, 2001; Corcoran et al., 2005; Sierra-Mercado et al., 2011). Accordingly, extinction of conditioned fear correlates with decreased c-Fos expression in the CeA and increased c-Fos expression in the IL cortex at extinction recall (Santini et al., 2004; Hefner et al., 2008). Thus, the increased and persistent c-Fos expression in the hippocampus and amygdala of Tg*NTRK3* mice identifies an abnormally active circuit that is likely causal for the observed changes in the fear responding of these animals.

Given the engagement of hippocampus in PAND (Charney, 2003; Massana et al., 2003a,b) and our results, the next experiments focused in this brain region. In the hippocampus, the glutamatergic and GABAergic neurotransmitter systems allow for dynamic and activity-dependent plastic functions, which are required for fear learning and memory processes. Strikingly, the analysis of excitatory (VGLUT1) and inhibitory (GAD65/67 and VGAT) neuronal markers suggests an imbalance in hippocampus of Tg*NTRK3* mice favoring hyperexcitability (Fig. 6*A*,*B*). In agreement with our observations of c-Fos induction, these changes mapped specifically to ventral CA1 hippocampal region (Fig. 6*C*,*D*). Interestingly, NT3 increases the probability of neurotransmitter release at the presynaptic terminal and promotes the establishment of functional excitatory synapses (Collin et al., 2001). However, no change was found in the number of excitatory synapses (VGLUT1/PSD-95) of Tg*NTRK3* animals (Fig. 6*E*), suggesting that changes in the efficacy of synaptic transmission and/or intracellular signaling may cause the altered learning-related activation of this region. In fact, in Tg*NTRK3* mice NMDA receptor expression and LTP in the hippocampus are increased (Sahún et al., 2007). Despite the increase in hippocampal NR2B expression, the NR2B antagonist ifenprodil did not improve contextual memory deficits in Tg*NTRK3* mice (Fig. 7*A*).

In fact, in a behavioral model of panic/fear, periaqueductal gray stimulation induces long-lasting fear-like response by deactivation of the GABAergic system (parvalbumin-positive interneurons) in the hippocampus (Temel et al., 2012), suggesting that the inhibitory system is actively involved in the fear process. Thrillingly, increasing inhibition through administration of tiagabine, both systemically and more importantly locally in the hippocampus, rescued the contextual fear memory phenotype of Tg*NTRK3* mice (Fig. 7*B*). This is interesting in light of findings that tiagabine treatment improves panic or agoraphobic symptoms in PAND patients (Zwanzger and Rupprecht, 2005). Additionally, these results highlight the importance of a proper hippocampal functioning, supporting our hypothesis of the differential involvement of this structure in PAND.

We here provide strong evidence that the hippocampus has a critical role in PAND, a brain region in which TrkC is highly expressed. The recovery of fear memory by tiagabine administered locally in the hippocampus, demonstrates the crucial role of this brain structure in the effectiveness of pharmacological intervention. These results open a new avenue of investigation of the neural mechanisms underlying PAND pathogenesis, leading to better understanding of the pathophysiology of this disorder and to the identification of new putative therapeutic entries. The con-

tribution of the neurotrophin receptor TrkC is probably extended to a broad range of anxiety disorders for which *NTRK3* susceptibility has been found.

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