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Behavioral phenotyping of Nestin-Cre mice: Implications for genetic mouse models of psychiatric disorders

Sebastian A. Giusti [a,1], Claudia A. Vercelli [b,1], Annette M. Vogl [a], Adam W. Kolarz [c], Natalia S. Pino [a], Jan M. Deussing [c], Damian Refojo [a,*]

[a] Molecular Neurobiology, Max Planck Institute of Psychiatry, Munich, Germany
[b] Instituto de Investigación en Biomedicina de Buenos Aires (IBioBA)-CONICET-Partner Institute of the Max Planck Society, Buenos Aires, Argentina
[c] Department of Stress Neurobiology and Neurogenetics, Max Planck Institute of Psychiatry, Munich, Germany

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ABSTRACT

Genetic mouse models based on the Cre-*lox*P system have been extensively used to explore the influence of specific gene deletions on different aspects of behavioral neurobiology. However, the interpretation of the effects attributed to the gene deletion might be obscured by potential side effects secondary to the Cre recombinase transgene insertion or Cre activity, usually neither controlled nor reported. Here, we performed a comprehensive behavioral analysis of endophenotypes of neuropsychiatric disorders in the extensively used Nestin^{Cre} mouse line, commonly employed to restrict genetic modifications to the CNS. We observed no alterations in locomotion, general exploratory activity, learning and memory, sociability, startle response and sensorimotor gating. Although the overall response to stimuli triggering anxiety-like behaviors remained unaltered in Nestin^{Cre} mice, a strong impairment in the acquisition of both contextual- and cued-conditioned fear was observed. These results underline the importance of adequately controlling the behavioral performance of the employed Cre-lines *per-se* in pre-clinical neurobehavioral research.

1. Introduction

Over the past 20 years, the use of targeted genetic modifications in mice has revolutionized our understanding of gene function and the way we investigate the pathophysiology of diseases. Knock-out (KO) and knock-in (KI) as well as transgenic strategies have been employed not only to uncover the physiological role of specific genes but also to develop animal models of human brain disorders, offering insights into anatomical, neurochemical, and behavioral effects of aberrant gene expression.

New technologies in molecular genetics have dramatically increased the number of targeted gene mutations available to the biomedical research community. Despite these advances, the difficulties of modeling disorders that often seem uniquely human still persist since they stem on intrinsic differences between species that cannot be overcome by mouse genetic manipulations. Thus, modeling human neuropsychiatric disorders in animals has been certainly challenging given the subjective nature of many symptoms, the lack of biomarkers and objective diagnostic tests, and our currently poor understanding of the etiological bases of complex brain diseases (Refojo and Deussing, 2012; Nestler and Hyman, 2010). A prevailing view is that animal models are unlikely to mirror the full extent of a given human neuropsychiatric disorder. Therefore, several experimental approaches aim to elucidate the basis of discrete symptoms clusters and physiological alterations that may be more amenable to genetic studies than the fully expressed psychiatric manifestation of a disease, with the assumption that what causes the symptom contributes mechanistically to the illness (Refojo and Deussing, 2012; Nestler and Hyman, 2010). This approach has involved the use of endophenotypes, defined as a set of behavioral and/or physiologic characteristics that accompany a basic process that is altered in relation to the illness under investigation (Gottesman and Gould, 2003; Tarantino and Bucan, 2000). In major depression, for instance, anhedonia, cognitive deficits, increased stress sensitivity and alterations in sleep architecture have been suggested as useful endophenotypes (Berton and Nestler, 2006; Refojo and Holsboer, 2009; Hasler et al., 2004) In the case of anxiety-related disorders, investigators have focused on endophenotypes reflected by fear or

* Corresponding author.
E-mail address: refojo@mpipsykl.mpg.de (D. Refojo).
[1] These authors equally contributed to this work.

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avoidance-related responses to either experienced (foot shock) or instinctively imprinted (aversion to highly illuminated arenas) threats. These singular behavioral traits are examined by behavioral paradigms such as conditioned fear and 'approach-avoidance'-based tasks, classical tests for scrutiny of anxiety-related behaviors (Leonardo and Hen, 2006; Cryan and Holmes, 2005; File et al., 2005).

Spatial and temporal control of genetic modifications has expanded and refined the toolbox of genetic modifications available to neuroscientists. The spatial and temporal control is usually achieved by conditional gene targeting based on the Cre-*lox*P system (Nagy, 2000; Branda and Dymecki, 2004). Bacteriophage P1-derived, site-specific, Cre recombinase is able to identify, bind and recombine DNA between two *lox*P sites (Sauer and Henderson, 1988). The basic strategy for Cre-*lox*P-directed gene knockout experiments is to flank, or "flox", an essential exon of the gene of interest with two *lox*P sites (Gu et al., 1994). Then, Cre excises the intervening DNA including the exon from the chromosome, thus generating a null allele in all cells where Cre is active. Delivery of Cre can be achieved by crossing mice carrying the floxed target gene with transgenic Cre-expressing mice. Thus, the spatial and temporal aspects of gene targeting will be dictated by the promoter driving the expression of Cre, since its regulatory sequences will primarily define the temporal and spatial specificity of the recombination and consequently of the gene ablation. As an additional layer of regulation, external temporal control of Cre activity can be achieved by using ligand-dependent chimeric Cre recombinases, such as CreER recombinases (Feil et al., 1996).

The advent of the Cre-*lox*P system represented a major breakthrough in the way to analyze the impact of specific genes on complex behaviors (Deussing, 2013; Gaveriaux-Ruff and Kieffer, 2007). The generation of multiple Cre lines by numerous laboratories and consortia has exponentially expanded the portfolio of available Cre-based lines to spatio-temporally restrict gene ablation in the brain (Taniguchi et al., 2011; Gerfen et al., 2013) (The Jackson Laboratory [www.jax.org], Mutant Mouse Regional Resource Centers [MMRRC, www.mmrrc.org]). Nevertheless, warnings about several technical pitfalls of Cre lines have been raised in the recent years. Thus, unexpected expression patterns of Cre transgenes, variability in the recombination efficiency and potential influences of the Cre on diverse cellular processes including toxicity, can induce effects *per-se* and introduce biases to the experimental readouts (Schmidt-Supprian and Rajewsky, 2007; Harno et al., 2013). As an additional confounding factor, alterations in gene expression can result from the integration of the Cre transgene. This can be due to a direct disruption of the sequences of targeted genes or by effects mediated by control elements present in the transgenic vector, like promoters or enhancers. Notably, the assumption that Cre lines will show an expression pattern of Cre recombinase highly analogous to that of the endogenous promoter and that high expression of Cre will be innocuous to the biology of the cell, has been one of the main oversights in the design of experiments involving the Cre-*lox*P system based-transgenic lines.

The Nestin^{Cre} transgenic line originally described by Tronche and colleagues has been designed to drive Cre expression in neuronal and glia cell precursors resulting in genomic recombination exclusively in the CNS (Tronche et al., 1999). For the generation of the line, a transgenic construct containing a Cre recombinase gene under the control of the promoter and enhancer present in the second intron of the rat nestin gene, was used. FISH analysis determined that the transgene insertion location is on chromosome 12. Nestin^{Cre} is probably the Cre line most extensively used in neurobiology. The mouse genome database list more than 480 published papers using this line (http://www.informatics.jax.org) (Blake et al., 2014) It has been employed in a variety of studies ranging from the analysis of neural development (Niola et al., 2012; Li et al., 2012; Moers et al., 2008) to the evaluation of psychiatric endophenotypes (Lu et al., 2008; Tronche et al., 1999; Zhao et al., 2008; Michan et al., 2010; Zhang et al., 2008; Refojo et al., 2011; Xu et al., 2010). However, to the best of our knowledge, a systematic characterization of potential alterations in behavioral outputs related to emotional responses has not been addressed so far.

Therefore, we aimed to analyze in the mentioned Nestin^{Cre} line, the contribution of the Cre transgene to the behavioral outcomes frequently used as endophenotypes of neuropsychiatric disorders. To this end we comprehensively tested in these mice, motor functions, learning and memory, social interactions, and traits relevant to anxiety, depression and schizophrenia.

2. Materials and methods

2.1. Animals

Nestin^{Cre} mice (Tronche et al., 1999) were maintained in a C57BL/6 background and bred in house. To obtain brain tissue, animals were handled according to the Guide for the care and Use of Laboratory Animals of Government of Bavaria, Germany. Animal experiments were done in accordance to local regulations and the NRC Guide for the Care and Use of Laboratory Animals followed at the IBioBA-CONICET and approved by the local IACUC.

2.2. In situ hybridization (ISH)

In situ hybridization (ISH) was performed as previously described (Refojo et al., 2011). Brains were carefully removed and immediately shock-frozen on dry ice. Frozen brains were cut on a cryostat in 20-μm thick sections and mounted on SuperFrost Plus slides. Specific riboprobes for Cre recombinase were generated by PCR applying T7 and T3 or SP6 primers using plasmids containing the above-mentioned cDNA as template. Radiolabeled sense and antisense cRNA probes were generated from the respective PCR products by *in vitro* transcription with ^{35}S-UTP using T7 and T3 or SP6 RNA polymerase.

Hybridization was performed overnight with a probe concentration of 7×10^6 c.p.m./ml at 57 °C and slides were washed at 64 °C in 0.1 × saline sodium citrate (SSC) and 0.1 M dithiothreitol. Hybridized slides were dipped in autoradiographic emulsion (type NTB2), developed after 2 weeks and counterstained with cresyl violet.

Dark-field photomicrographs were captured with digital cameras adapted to an imaging microscope and a stereomicroscope. Images were digitalized using Axio Vision 4.5, and afterward photomicrographs were integrated into plates using image-editing software. Only sharpness, brightness and contrast were adjusted. For an adequate comparative analysis in corresponding control and Nestin^{Cre} sections the same adjustments were undertaken. Brain slices were digitally cut out and set onto an artificial black background.

2.3. Behavioral studies

In all behavioral experiments male mice were used, aged 8–12 week, single housed two weeks prior to the experiment, under standard laboratory conditions (22 ± 1 °C, 55 ± 5% humidity) with food and water *ad libitum*. Littermates were used for the analysis.

Behavioral experiments were performed with two independent batches of mice. Batch 1 underwent a battery of tests consisting of the Y-maze, open-field test (OF) under aversive conditions, sociability test and the elevated plus-maze test (EPM). All tests were performed in the order listed between 9 a.m. and 12 a.m. The

animals' behavior during the tests was videotaped (sociability test) and scored by a trained observer blind to the animals' genotype using 'Eventlog' (version 1.0, Emco Software Ltd., Reykjavik, Iceland) or was automatically analyzed (for the Y-maze, OF and EPM) by tracking the 'center of the animal' using the 'ANY-maze' video-tracking software (Stoelting Co., Wood Dale, Illinois, USA).

Mice from batch 2 were kept in a reversed 12 h:12 h light–dark cycle with lights off at 9 a.m. Behavioral experiments were conducted during the activity phase of the animals. Batch 2 underwent a battery of tests consisting of OF performed in the dark, water cross maze, radial maze, acoustic startle response (ASR), prepulse inhibition (PPI), tail suspension test (TST) and fear conditioning. All tests were performed in the order listed.

2.3.1. Open field in the dark

The OF test was performed to measure locomotor activity and general exploratory behavior by using the TruScan Photo Beam Activity system (Coulbourn Instruments, Whitehall, PA, USA) Mice were put onto the center of a Plexiglas cage (L26 × W26 × H38 cm, 0 Lux) for 30 min testing. Each test cage, including the sensor rings, was surrounded by walls made of opaque Plexiglas (L47 × W47 × H38.5 cm). Horizontal and vertical locomotion were automatically recorded by 2 photobeam sensor rings (2 and 5 cm above the floor; photobeams are spaced apart by 1.52 cm providing a 0.73 cm spatial resolution). The distance traveled and mobility times were recorded and analyzed (sampling rate 4 Hz) by TruScan Software Version 1.1 (Coulbourn Instruments). For analysis, the test duration was subdivided into individual segments of 5 min each. Animals were removed from the test cages in the end of the test and Plexiglas cages were cleaned with detergent-containing water.

2.3.2. Radial maze

Short-term working memory was assessed with the radial maze, which consists of 8 horizontal arms (57 × 11 cm), placed radially around a central platform. The test room is illuminated with dim light (15 lux) and rich in extra maze visual cues. Mice were placed in the platform of the maze and the entry into each of the arms was scored. Re-entries into the arm previously visited are counted as an error entry. One trial continues until mice complete the entries into all arms and errors are counted (cutoff time: 10 min). Each mouse performs one trial per day, for 4 consecutive days. To prevent odor cues, the maze was wiped clean between animals.

2.3.3. Water-cross maze

The water-cross maze (WCM) was implemented to assess spatial memory performance, and was performed as previously described (Kleinknecht et al., 2012). Similarly to the classical Morris Water Maze, the WCM makes use of water-based motivation. The maze consists of two intersecting arms, forming a cross, made from clear acrylic glass to enable visual orientation within the room. A submerged platform was located in one on of the arms, 1 cm under the water surface, invisible to the mice. Every animal performed six trials a day for five consecutive days. During this time the platform was always located in the same arm (ex. East), whereas the starting position of the mice alternated between South and North in a pseudorandom manner. The latency to reach the platform was set to 1 min. Learning performance was assessed by accuracy: A trial was considered accurate, if the animal directly entered the arm containing the platform and climbed onto it. Aberrant behavior was considered as non-accurate. Thus, accuracy reflects the percentage of accurate trials on each day per animal.

2.3.4. Y-maze

Spatial memory was investigated with the Y-Maze, which consisted of three evenly illuminated arms (15 lux), each marked by a distinct intra-maze cue (triangle, bar or plus sign). The test included an acquisition and retrieval stage, separated by a 30-min inter trial interval (ITI). During the acquisition stage, one of the arms was blocked, allowing the mice to freely explore the other two arms for 10 min. During the retrieval stage, the mice were allowed to freely explore all three arms for another 10 min. Learning performance was successful, if the time spent in the novel arm compared to the known arms, was significantly higher than chance level (>33.3%).

2.3.5. Sociability test

The sociability test was performed using a three chamber apparatus, as previously described (Nadler et al., 2004; Hartmann et al., 2012; Moy et al., 2004). An unfamiliar male C57BL/6J mouse was introduced into the left chamber, enclosed in a wire cage; while a toy mouse was placed in the opposite chamber (alternation occurred after 3 consecutive trials). The time spent interacting with mouse and object was scored for 10 min by a trained observer.

2.3.6. Open field in the light

Testing was performed in open field boxes (50 × 50 × 40 cm) made up of gray polyvinyl chloride (PVC) evenly illuminated with 15 lux. The test duration was 30 min, subdivided into individual segments of 5 min each. The total distance traveled and mobility, and the distance traveled in the more aversive inner zone (30 cm diameter) of the OF was measured.

2.3.7. Elevated plus maze

Anxiety-related behavior was measured by means of the EPM. The apparatus was made of gray PVC and consisted of a plus-shaped platform with four intersecting arms, elevated 37 cm above the floor. Two opposing open (30 × 5 cm) and closed arms (30 × 5 × 15 cm) were connected by a central zone (5 × 5 cm). Animals were placed in the centre of the apparatus facing the closed arm and were allowed to freely explore the maze for 5 min. Parameters of interest included open arm time, distance traveled in the open arm and open arm entries.

2.3.8. Tail suspension test

The details of tail suspension test (TST) are described previously (Steru et al., 1985). Each tested animal was suspended by the tip of tail with an adhesive tape to a rod that was 35 cm above the ground for 6 min. Four animals were tested at the same time. Each trial was videotaped and the immobility time was analyzed by an observer blind to the mouse line or treatment using the computer software 'Eventlog' (version 1.0, Emco Software Ltd., Reykjavik, Iceland).

2.3.9. Acoustic startle response and prepulse inhibition

To estimate the arousal state of the animals, acoustic startle responses were measured in the following way: mice were placed into one out of seven identical startle set-ups, consisting of a non-restrictive Plexiglas cylinder (inner diameter 4 cm, length 8 cm) mounted onto a plastic platform, each housed in a sound attenuated chamber (SR-LAB, San Diego Instruments SDI, San Diego, CA, USA). The cylinder movement was detected by a piezoelectric element mounted under each platform and the voltage output of the piezo was amplified and then digitized (sampling rate 1 kHz) by a computer interface (I/O-board provided by SDI). The startle amplitude was defined as the peak voltage output within the first 50 ms after stimulus onset and quantified by means of SR-LAB software. Before startle measurements, we calibrated response sensitivities for each chamber in order to assure identical output levels. Startle stimuli and background noise were delivered through a high-frequency speaker placed 20 cm above each cage. The 4 different startle stimuli consisted of white noise bursts of 20 ms

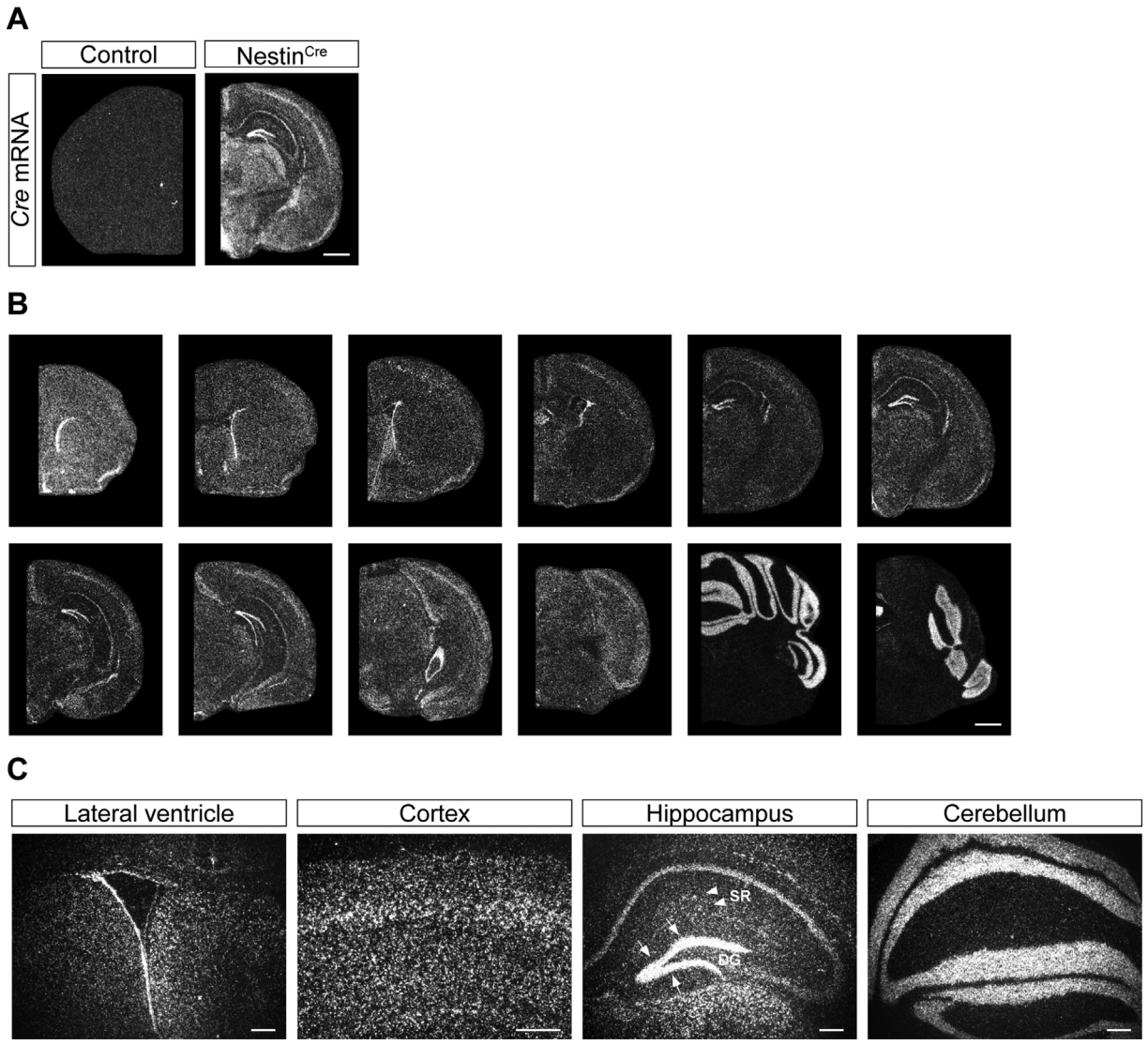

Fig. 1. Cre expression in the brain of Nestin^{Cre} adult mice. The expression of Cre was analyzed by *in situ* hybridization using a specific radiolabeled riboprobe detecting Cre. (A) The specificity of Cre signal was confirmed using Cre negative littermate mice as control. Scale bar: 1 mm. (B) Cre is expressed throughout the anterior–posterior axis of the adult mouse brain. Scale bar: 1 mm. (C) Details of the expression of Cre in different brain regions. SR: *stratum radiatum* (arrowheads). DG: dentate gyrus (arrows). Scale bar: 250 μm.

duration and 75, 90, 105 and 115 dB intensity were presented in a constant background noise of 70 dB. Intensity was measured using an audiometer (Radio Shack, 33-2055, RadioShack, Fort Worth, TX, USA). On control trials only background noise was present. After an acclimation period of 5 min duration, 16 control trials and 30 startle stimuli of each intensity were presented in pseudorandom order in each test session. The interstimulus interval was 15 s averaged (13–17 s, pseudorandomized). Plexiglas cylinders were cleaned thoroughly with soap water after each trial.

The prepulse inhibition (PPI) of the ASR was performed in a different day. In this case, startle stimuli (110 dB, 50 ms) were presented alone, or preceded by noise prepulses (20 ms) of 2, 4, 8, or 16 dB above background (70 dB), with 100 ms between onsets of the prepulse and startle stimuli. The test session started with a 5-min acclimation period followed by three consecutive blocks of test trials (block 1 and 3, startle-stimulus alone trials; block 2, startle-stimulus alone, startle + prepulse, and no-stimulus trials). The interstimulus interval was 15 s averaged (13–17 s, pseudorandomized). PPI was calculated as follows: % PPI = [(PP-condition − pulse-alone)/pulse-alone × 100%].

2.3.10. Fear conditioning

Contextual and auditory fear memory were assessed as previously described (Refojo et al., 2011; Kamprath and Wotjak, 2004). Two different contexts were used for the experiments. Foot shoot delivery and context-dependent fear memory were assessed in a cubic-shaped chamber with metal grid floors. A neutral context consisting of a Plexiglas cylinder with bedding was used to investigate auditory (tone-dependent) fear memory. For foot shock application (day 0) mice were placed into the conditioning chamber for 3 min. After 180 s, a sine wave tone (80 dB, 9 kHz) was presented for 20 s, which co-terminated with a 2 s scrambled electric foot shock of 0.74 mA. The mice remained in the conditioning chamber for another 60 s. Contextual (associative) fear was tested by re-exposing the animals to the conditioning grid chamber for 3 min on day 1. In order to measure freezing responses to the tone, mice were placed into the neutral environment (cylinder) on the following day (day 2). Three minutes later, a 3 min tone was presented (80 dB, 9 kHz). The animals were returned to their home cages 60 s after the end of tone presentation. As a measure of fear, freezing behavior was recorded and analyzed by an observer blind to genotype.

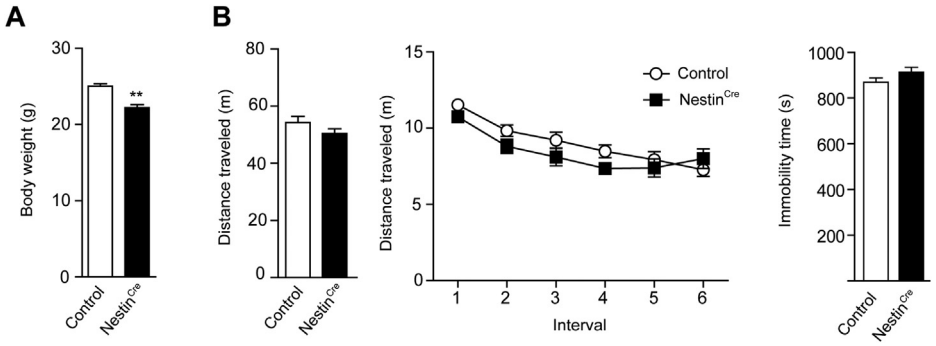

Fig. 2. Body weight and locomotor behavior. (A) Nestin^{Cre} mice are significantly lighter than littermate controls. (B) Total distance traveled and immobility (%) in the open field during the 30 min test period did not differ between the groups. Values represent mean + s.e.m. Student's *t* test, $^{*}p < 0.05$.

3. Results

3.1. Cre expression in Nestin^{Cre} adult mouse brain

The reason why the nestin locus has been used to target genes in a CNS-specific manner stems on the fact that the nestin gene is highly transcribed in neural progenitors that will give rise to neurons and macroglial cells (astrocytes and oligodendrocytes) of the CNS.

Endogenous nestin expression is downregulated when CNS progenitor cells differentiate into neurons or glial cells (Lothian and Lendahl, 1997). Nevertheless, a systematic analysis of Cre recombinase expression throughout the adult brain of Nestin^{Cre} mice has not been reported so far. Therefore we mapped the Cre mRNA expression pattern in the Nestin^{Cre} brain by *in situ* hibridization (ISH).

In contrast to the reported expression pattern of the endogenous nestin gene, we observed a widespread expression of Cre recombinase in the adult brain, driven by the transgenic rat nestin promoter/enhancer (Fig. 1A, B). Neuronal expression is consistent with the presence of the signal in cortical layer II/III, pyramidal cells of the hippocampus and the granule cell layer of the cerebellum (Fig. 1C). In addition, astroglial expression can be inferred by the presence of the signal in the *stratum radiatum* of the hippocampus (Fig. 1C). Notably, high expression of Cre was detected in neurogenic areas of the brain, such as the lateral wall of the lateral ventricle and the dentate gyrus (Fig. 1C). These areas are known to express endogenous nestin in neuroprogenitor cells of the adult mouse brain (Lagace et al., 2007; Mignone et al., 2004; Kawaguchi et al., 2001).

3.2. Body weight and locomotor activity

Adult Nestin^{Cre} mice were significantly lighter (11.2%) than littermate controls (Fig. 2A).

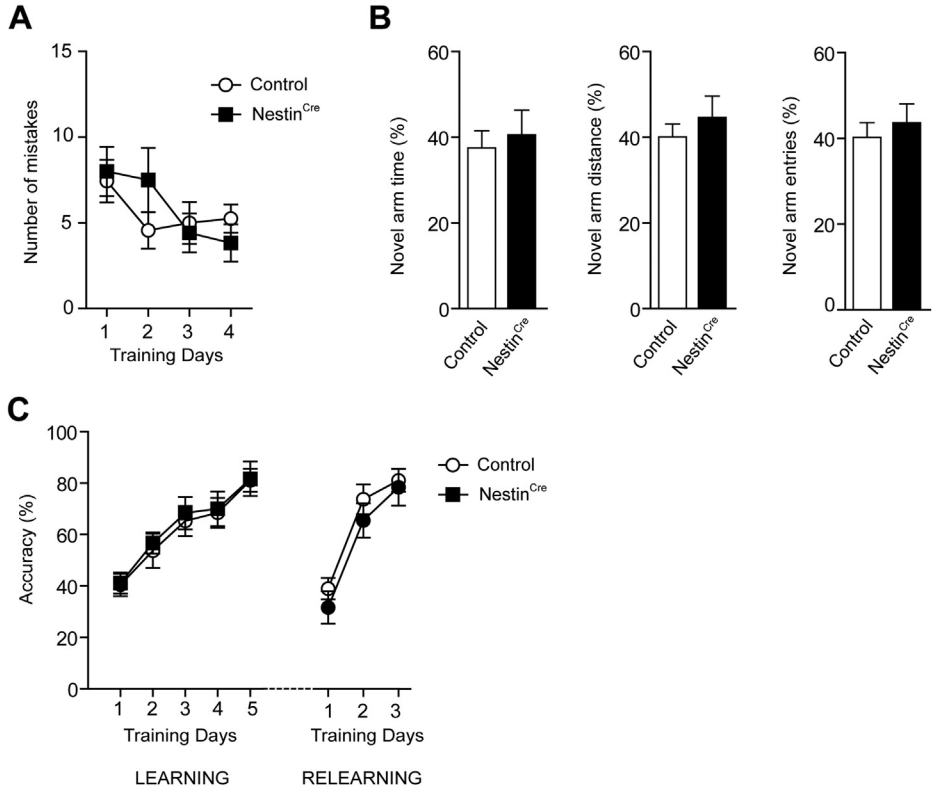

Fig. 3. Learning and memory. No differences in the performance of Nestin^{Cre} vs. littermate controls were observed in (A) the radial arm maze, (B) Y-maze or (C) the water cross maze. Values represent mean + s.e.m. (A, C) Repeated measures two-way ANOVA. (B) Student's *t* test.

Most behavioral tests assessing cognitive abilities or emotional responses are based on tasks that require normal locomotor skills and exploratory traits, which are then the first control tests usually explored in the phenotyping of emotional behavior.

Locomotion and general exploratory behavior were assessed with the open field test performed under non-aversive illumination conditions. No differences between genotypes were found between Nestin^{Cre} and control littermates indicating that Cre insertion and expression in Nestin^{Cre} mice does not influence these two relevant parameters (Fig. 2B).

3.3. Learning and memory

Learning and memory deficits are relevant endophenotypes of several psychiatric disorders including major depression, schizophrenia and bipolar disorder (Millan et al., 2012). Therefore, we comprehensively compared the performances of Nestin^{Cre} mice and control littermates in a set of behavioral tests that are frequently used to investigate cognitive functions in animal models. Short-term working memory was assessed with the radial maze (Fig. 3A) while spatial working memory was evaluated with the Y-maze (Fig. 3B). In addition, we analyzed spatial reference memory with the water cross maze (Fig. 3C). Using this paradigm, spatial learning flexibility was assessed during the relearning phase. It is worth to underline that the behavioral performance in these tests relies not only on hippocampal function; the septum and prefrontal cortex are also crucial for an appropriate performance in the Y-maze and radial maze paradigms. Therefore, the normal execution of these tests, indicate that not only the hippocampal control but also the function of other limbic areas in cognitive responses are preserved in Nestin^{Cre} mice.

3.4. Sociability

A variety of neuropsychiatric disorders, including depression and anxiety, are characterized by disruptions of social behavior (Nestler and Hyman, 2010; Berton and Nestler, 2006). In particular, sociability tests are often used to explore autistic-like behavior in mice, since deficits in social interaction are important early markers for the autistic-spectrum and related neurodevelopmental disorders (Moy et al., 2004; Moretti et al., 2005). We found no differences in social tendency between groups, measured as the percentage of time of interaction with a novel conspecific compared to the time of interaction with an inanimate toy mouse (% social interaction, control: 61.2 $\pm$ 3.2%; Nestin^{Cre}:56.3 $\pm$ 3.8%, $p = 0.34$, Student's t test). Both groups showed a normal preference for the interaction with the social target over the inanimate target.

3.5. Anxiety-related behaviors

In an attempt to model human pathological anxiety in rodents, a wide range of behavioral testing paradigms have been developed (Bourin et al., 2007). Many of these tests integrate an approach–avoidance conflict designed to inhibit an ongoing behavior that is characteristic for the animal, such as contrasting the tendency of mice to engage in exploratory activity against the aversive properties of an open, brightly lit, or elevated space. From this kind, we employed the open field under illuminated conditions and the elevated plus maze (EPM). We observed that distance traveled in the more aversive inner zone of the open field and the immobility time were not different for Nestin^{Cre} and control mice (Fig. 4A). On the other hand, Nestin^{Cre} mice spent less time in the open arms of the EPM, pointing towards an increased anxiety phenotype of these mice (Fig. 4B). Nevertheless other classical parameters of the test such as distance traveled in the open arm and number of entries into the open arm show no differences between genotypes suggesting that anxiety-related behavior was not overall affected in Nestin^{Cre} mice.

The tail suspension test (TST) has been originally described to screen the activity of monoamine-based antidepressant compounds. Under basal conditions, the TST has been frequently considered as a test evaluating depression-like behavior (Berton et al., 2012). From our perspective and the one of others researchers in the field (Refojo and Deussing, 2012; Nestler and Hyman, 2010) there is not sufficient validation supporting this claim and the test does not have clear face and construct validity for depression. However, the test involves a stress component that can be used to evaluate coping strategies against inescapable stress. Under this view the immobility strategy in the tail suspension test, homologous to the immobility behavior of the forced swim test, represent passive stress-coping behavior, which was similar in both Nestin^{Cre} and littermate controls (Fig. 4C).

The acoustic startle response (ASR) is a protective response, elicited by a sudden and intense acoustic stimulus. Facial and skeletal muscles are activated within a few milliseconds, leading to a whole body flinch in rodents. The use of the ASR as a behavioral measure of fear/anxiety has fewer confounds relative to more classic conflict tests, such as open field and EPM, which use locomotor activity and approach/avoidance conflicts to assess emotional responding in animals (Dulawa et al., 1999). We found no differences in the strength of the ASR between genotypes in any of the tested stimulus intensities (Fig. 4D), indicating similar arousal levels in both groups of mice. We also observed no differences in sensorimotor gating, measured using prepulse inhibition (PPI) of the ASR (Fig. 4E), a behavioral modality that is disrupted in schizophrenia patients and animal models (Braff et al., 2001).

Fear conditioning, a basic form of associative learning, is a traditional method for the study of etiological processes related to fear and anxiety (LeDoux, 1998). Interestingly, we found a significant reduction in the freezing response to the context (hippocampus-dependent) and tone (amygdala-dependent) of Nestin^{Cre} mice, scored one and two days after the conditioning, respectively. These results indicate an impairment in the acquisition of both contextual and cued fear in Nestin^{Cre} compared to control littermates (Fig.5A, B).

4. Discussion

In this study we performed a comprehensive behavioral phenotyping of the extensively used Nestin^{Cre} mouse line (Tronche et al., 1999), with focus in endophenotypes of neuropsychiatric disorders. We found that the presence of the Cre recombinase transgene does not interfere with most tests assessing emotional behavior. However, we found a strong impairment in the acquisition of conditioned fear in Nestin^{Cre} mice.

Although we did not test the potential causes underlying the behavioral phenotype of Nestin^{Cre} mice, transgene insertion and Cre activity have been previously associated with side effects (Schmidt-Supprian and Rajewsky, 2007). Numerous reports have shown that Cre activity can cause toxicity, even in the absence of a "floxed" allele, regardless whether its expression is ubiquitous, tissue-specific and/or ligand-inducible (Jeannotte et al., 2011; Schmidt et al., 2000; Naiche and Papaioannou, 2007; Huh et al., 2010; Lee et al., 2006). Cre toxicity was shown to result in growth arrest, chromosomal abnormalities, and apoptosis (Loonstra et al., 2001; Thanos et al., 2012; Janbandhu et al., 2014). Unlike the expression pattern of the endogenous nestin gene, characterized by a progressive downregulation during the postnatal period (Ernst and Christie, 2005), we found that Cre mRNA was abundant in the adult brain of Nestin^{Cre} mice. This extended period of Cre activity, raises the possibility that Cre expression during adulthood might play a role in the phenotype of Nestin^{Cre} mice.

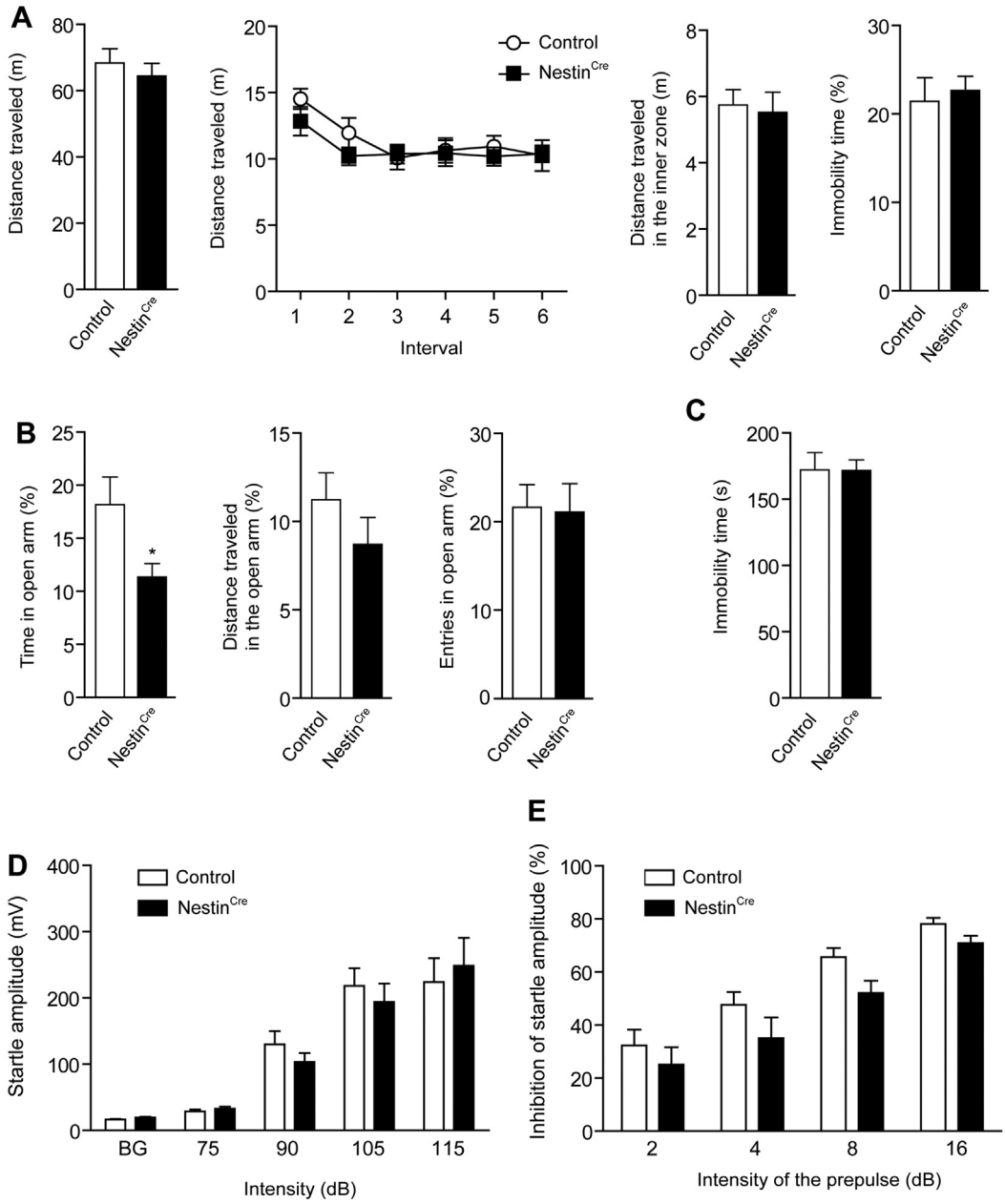

Fig. 4. Anxiety-related behavior. (A) Total distance traveled, distance traveled in the aversive inner zone and immobility (%) in the open field during the 30 min test period, did not differ between the groups. (B) Nestin^{Cre} mice spent significatively less time (%) in the open arms of the elevated plus maze than controls. No differences were found in the number of entries (%) or distance traveled (%) in the open arms. (C) Immobility time during tail suspension test did not differ between groups. (D) Acoustic startle response elicited by acoustic stimuli. BG: background sound intensity was 70 dB. (E) Prepulse inhibition of the startle response was not different between the groups. Intensity values represent dB above background. Values represent mean + s.e.m., $^{*}p < 0.05$. (A–C) Student's *t* test. (D, E) Repeated measures two-way ANOVA.

The relevance for the study of this particular Cre line is emphasized by a comparative analysis revealing that among the 26 available Nestin^{Cre} transgenic lines, the one developed by Tronche and colleagues has been used in 487 studies, while the rest of the lines together are cited by 256 reports. This extensive use is also evident when compared to other commonly used, lines, such as CamK2a^{Cre} (35 lines, 3714 references) or Neurod6/Nex^{Cre} (4 lines, 81 references) (Mouse Genome Database (MGD) at the Mouse Genome Informatics website, The Jackson Laboratory, Bar Harbor, Maine. URL: http://www.informatics.jax.org. [March, 2014]) (Blake et al., 2014).

We observed that Nestin^{Cre} mice have smaller body weights as previously reported (Karaca and Maechler, 2014; Briancon et al., 2010). This is probably related to the fact that this Cre line is affected by mild hypopituitarism (Galichet et al., 2010). Regarding the behavioral phenotype, we observed no alterations in locomotion, general exploratory activity, learning and memory, sociability, startle response and sensorimotor gating. However, we found a strong impairment in the acquisition of conditioned fear. Notably, some studies using this line to knockout different genes in the CNS, have reported a similar impairment in the freezing to the tone or the context after fear conditioning (Gao et al., 2010; Suzuki et al., 2013), attributing the effect to the deleted gene. Following an extended practice, these studies used "floxed" mice as a control group and did not include a control for the Cre transgene. Since behavioral outcomes are sensitive to the genetic background and

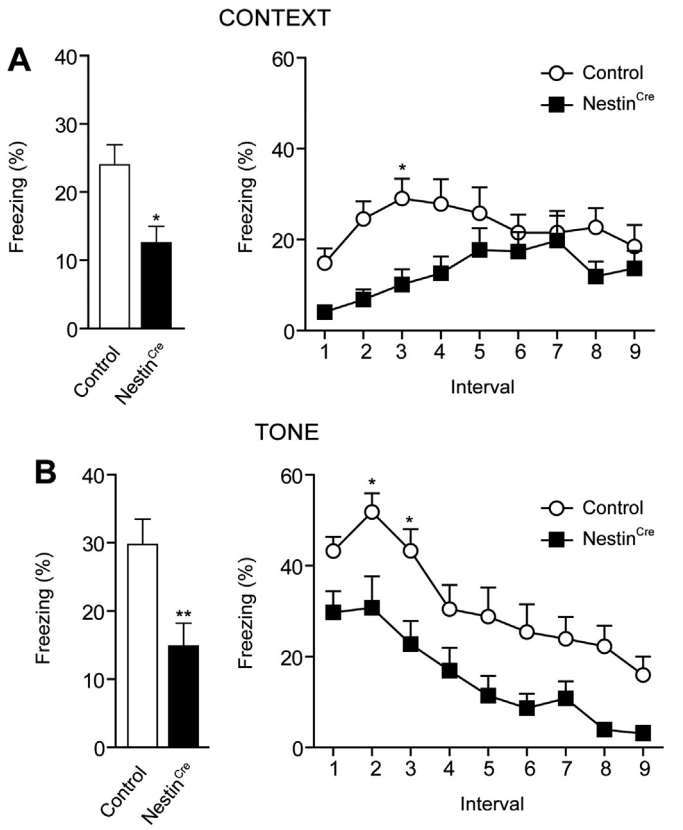

Fig. 5. Acquisition of conditioned fear. During the conditioning process, mice were placed in a cubic-shaped conditioning chamber with a metal grid and an auditory tone was paired to an electrical foot shock. Freezing responses to the context (24 h later) (A) and to the tone (48 h later) (B) were significantly reduced in Nestin^{Cre} mice compared to littermate controls. Values represent mean + s.e.m., $^{**}p < 0.01$, $^{*}p < 0.05$. Student's *t* test for the total freezing time (left) and repeated measures two-way ANOVA followed by Bonferroni post-test for the interval (20 s) analysis (right).

environmental factors including handling, noise levels and season (Crabbe et al., 1999; Crawley, 2008), our observations do not invalidate the conclusions of the mentioned reports, but remark the importance of including appropriate controls to accurately interpret results of gene targeting studies.

In summary we describe here for the first time a behavioral characterization of the most extensively used Cre mouse line in neurobiology and psychiatric research: the Nestin^{Cre} line originally developed by Tronche and colleagues. Importantly, our results indicate that indirect effects from the process used to create the null mutation can be confused with the effects of the induced mutation itself. Therefore, our work highlights the importance of including relevant experimental groups to control for the potential effects of the Cre transgene insertion.

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Contributors

S.A.G., C.A.V., A.M.V., A.W.K., N.S.P., performed the experiments and analyzed the data. J.M.D. analyzed the data, interpreted the results and edited the manuscript. D.R. supervised the project and wrote the manuscript together with S.A.G.

Conflict of interest

The authors report no conflict of interest or disclosures of financial interests and relationships, and have not received any payment for the preparation of this manuscript. The authors alone are responsible for the content and writing of the paper.

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