Introduction

In mammals, neurons producing serotonin (5-hydroxytryptamine, 5-HT) are generated early during embryonic development in the ventral hindbrain and progressively cluster into B1–B9 raphe nuclei that project to the whole central nervous system (CNS), from the anterior brain to the spinal cord [1,2,3]. The extensive and capillary organization of serotonergic terminals together with the existence of at least 15 different 5-HT receptors distributed in the CNS accounts for the multitude of physiological and behavioural functions mediated by brain serotonin, from the regulation of circadian rhythms [4] and mood [3], to social interaction [6] and sexuality [7]. Moreover, in addition to its function in neurotransmission, growing evidences support a role for serotonin in developmental processes as cellular proliferation, migration, neuronal differentiation and brain circuitry formation [8–12]. Consistently, an altered serotonergic signalling has been associated with neuropsychiatric disorders in humans thought to have neurodevelopmental basis, such as schizophrenia and autism [13,14].

During the last years, growing efforts have been made to generate suitable genetic tools to target serotonergic neurons in order to study their development and function [15–18]. Advances in mouse molecular genetics have brought new insights into the comprehension of the molecular cascade involved in serotonergic neuron specification [19–24], as well as of the projection network of raphe nuclei [11,25]. Altogether, these studies have been crucial to reconsider serotonergic system, rather than a homogenous ensemble, a complex and heterogeneous population with distinct morphological, molecular and electrophysiological characteristics [22,24–29].

The transcriptional pathways of serotonergic neuron differentiation require a combination of secreted molecules and transcription factors such as Shh, Fgf4/8, Nkx2.2, Lmx1b and Pet1. In the mouse, Pet1 mRNA expression appears between 10 and 11 days post coitum (dpc) in serotonergic post-mitotic precursors and persists in serotonergic neurons up to adulthood, where it promotes the expression of genes defining the mature serotonergic phenotype such as tryptophan hydroxylase 2 (Tph2) and serotonin transporter (SERT). Hence, the generation of genetic tools based on Pet1 specific expression represents a valuable approach to study the development and function of the serotonergic system. Here, we report the generation of a Pet1-Cre transgenic mouse line in which the Cre recombinase is expressed under the control of a 210 kb fragment from the Pet1 genetic locus to ensure a reliable and faithful control of somatic recombination in Pet1 cell lineage. Besides Cre-mediated recombination accurately occurred in the serotonergic system as expected and according to previous studies, Pet1-Cre transgenic mouse line allowed us to identify novel, so far uncharacterized, Pet1 expression domains. Indeed, we showed that in the raphe Pet1 is expressed also in a non-serotonergic neuronal population intermingled with Tph2-expressing cells and mostly localized in the B8 and B9 nuclei. Moreover, we detected Cre-mediated recombination also in the developing pancreas and in the ureteric bud derivatives of the kidney, where it reflected a specific Pet1 expression. Thus, Pet1-Cre transgenic mouse line faithfully drives Cre-mediated recombination in all Pet1 expression domains representing a valuable tool to genetically manipulate serotonergic and non-serotonergic Pet1 cell lineages.
converge on the activation of the ETS transcription factor Pet1 (plasmacytoma expressed transcription factor 1, official name Fev) [24,30]. The onset of Pet1 expression in the mouse has been described to occur approximately as early as 10.5 dpc in postmitotic precursors within the mantle layer, in the rostral hindbrain from rhombomere (r) 1 to r3, (i.e. rostral serotonergic domain), and one day later in r3-r7 (i.e. caudal cluster) [30–32]. In these domains Pet1 expression precedes the appearance of markers of serotonergic terminal differentiation, such as tryptophan hydroxylase 2 (Tph2) and serotonin transporter (SERT), and is maintained up to adulthood [30].

Thus, thanks to its early and specific expression, Pet1 represents the ideal candidate gene to be used in Cre recombinase/loxP-based strategies to specifically target serotonergic neurons. Such an approach represents a powerful tool to map the genetic lineage of Pet1 expressing cells and to characterize the molecular identity of distinct subpopulations of serotonergic neurons through intersectional strategies [33]. Moreover, a Cre recombinase-expressing line targeting serotonergic neurons could be used in conditional knock out approaches to investigate the involvement of specific genes in the development and functioning of serotonergic system, as well as in studies aimed to map serotonergic structural and functional connections in the brain.

In this context, the availability of BAC (Bacterial Artificial Chromosome)-based homologous recombination in E. coli approach allows the generation of large transgenic constructs, thus providing the presence of long distance acting regulatory elements required for the proper temporal- and tissue-specific expression of the gene of interest as well as reducing positional effect, that otherwise might drive transgene expression outside promoter-specific cell populations [34–39].

In the present study we used an E. coli homologous recombination based-approach to generate a Pet1210-Cre transgenic mouse line, in which 210 kb of Pet1 locus drive the expression of the Cre recombinase. Our analysis showed that in Pet1210-Cre mice Cre-mediated somatic recombination specifically occurs in serotonergic neurons of the raphe nuclei. Moreover, we demonstrated that Pet1 is expressed also in a population of non-serotonergic neurons within the raphe nuclei and in non-neuronal districts such as the ureteric bud derivatives of the kidney and the pancreas, starting from 9.5 dpc and 11.5 dpc, respectively. The Pet1210-Cre transgenic mouse line thus widens our knowledge on Pet1 gene expression and represents a valuable tool to promote Cre-mediated somatic recombination both in serotonergic and non-serotonergic Pet1 cell progeny.

Materials and Methods

Animals

Mice were housed in standard Plexiglas cages at constant temperature (22±1°C) and maintained on a 12/12 h light/dark cycle, with food and water ad libitum. Experimental protocols were conducted in accordance with the Ethic Committee of the University of Pisa and approved by the Veterinary Department of the Italian Ministry of Health.

Generation of the Pet1210-Cre transgenic mouse line

To generate the BAC-Cre construct we took advantage of a recombination-based strategy carried out in bacteria. To this aim we obtained a Pet1210-Cre targeting vector containing both the Cre recombinase cDNA and a kana/Neo resistance gene flanked by two 500 bp long homology arms. Briefly, Pet1210-Cre left arm (Pet1210LA) has been cloned in frame with Cre recombinase using an overlapping Polymerase Chain Reaction (PCR)-based strategy [40]. Briefly, Pet1210LA and Cre recombinase have been separately amplified by means of PCR using BAC_RP23_165D11 and a pSG5- Cre plasmid as templates, respectively. Amplification of Pet1210LA was performed using the following primers: forward 5’ ATTAT1CTCGAGGGGAGGTAGAAAAGGCAGCATTA 3’, reverse 5’ TTGGTGTTACGGCTACTTTATAAATTCGAGCTTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTGGTCTCAGCTGTCGGGCACTG
with Avertin and perfused intracardially with 4% PFA. Brains were dissected out, post-fixed o/n in PFA at 4°C and embedded in either 2.5% agarose or tissue-tek for sectioning with vibratome or cryostat, respectively.

For immunostaining experiments were incubated with primary antibodies o/n at 4°C in PBS containing 5% heat-inactivated lamb serum and 0.5% Triton X-100. Primary antibody dilutions: rabbit anti-5-HT (Sigma) 1:500; chicken anti-cGFP/cYFP (Abcam) 1:1000; mouse anti-calbindin-D-28K (Sigma), 1:200. Fluorescent-conjugated secondary antibody were used as follow: Rhodamine Red-X goat anti-rabbit IgG 1:500; Alexa Fluor 488 goat anti-chicken IgG 1:200; Rhodamine Red-X goat anti-mouse IgG 1:500 (all by Molecular Probes). Cell nuclei were counterstained with DAPI (Sigma), 0.5 μg/ml.

**X-gal chromogenic reaction**

X-gal staining was performed on 9.5 dpc, 10.5 dpc, 11.5 dpc, 13.5 dpc Pet1210-Cre/ROSA26R whole embryos and on P1 or P10, P30 and adult Pet1210-Cre/ROSA26R kidney and brains, respectively. Dissected tissues or whole embryos were fixed in 2% formaldehyde solution prepared in PBS for 90 minutes, and subsequently processed for X-gal staining solution containing 5 mM K4Fe(CN)6, 5 mM K3Fe(CN)6, 2 mM MgCl2, 0.2% NP40, 0.1% sodium deoxycholate and 1 mg/ml X-gal (Sigma) in PBS for 4–16 h at 30°C. Samples were post-fixed in 4% PFA at 4°C o/n.

β-galactosidase stained specimens were cut at 50 μm with a vibratome or clarified in methyl salicylate pure solution to enhance contrast between X-gal staining and non-stained tissues.

**In situ hybridization**

In situ hybridization was performed as previously described [11]. Briefly, animals were sacrificed by cervical dislocation and fresh brain tissue was dissected out, embedded in Tissue Tek (Sakura), frozen on dry ice and stored at −80°C until used. 14 μm cryostat sections were cut and hybridization was performed according to protocols using either digoxigenin- or fluorescein- or 33S-labelled antisense RNA probes. In digoxigenin-labelled in situ hybridization experiments, NBT/BCIP (Roche) was used as substrate for alkaline phosphatase, while in radioactive in situ hybridization sections were exposed to Biomax MR X-ray films (Kodak) for two to seven days. For double ISH, sections were hybridized simultaneously with DIG- and fluorescein-labelled probes. A two-step chromogenic reaction using NBT/BCIP and HNPP/Fast Red Fluorescent Detection Set (Roche) was performed to visualize DIG- and fluorescein-labelled riboprobes. Specimens were counterstained with DAPI.

**Image acquisition and data analysis**

For brightfield acquisitions, both sections and whole mount samples were observed and photographed with a light microscope or with a MacroFluo microscope equipped with DS-SMC digital cameras (Nikon). Fluorescence images were taken with Eclipse Ti microscopes (Nikon) or with a SP5 confocal microscope (Leica), using 10x and 63x objectives.

For cell counting, double ISH experiments were performed on three distinct C57BL/6j wild-type animals. Tph2- and Pet1-positive neurons in B9, B8, B7, B5–B6 and B1–B3 raphe nuclei were counted using ImageJ software. On average three to four sections depending on the antero-posterior extension of each nucleus were examined. In order to avoid counting cells twice, serial sections 70 μm distance one from another were analysed. For each section two to four 10x images were captured both in brightfield and in TRITC channel to visualize NBT/BCIP, DIG-labelled, or Fast Red, fluorescein-labelled, positive neurons, respectively. Images were converted to 8-bit greyscale and a threshold function was manually applied to remove sub-threshold signal using ImageJ software. For each image, only cells showing labelling clearly above the background level were counted. Pet1+/Tph2+ and Pet1+/Tph2− neurons were then counted per each raphe nucleus and the obtained values were expressed as relative percentages.

**Results**

**Generation of a Pet1210-Cre transgenic mouse line driving Cre-mediated recombination in the raphe nuclei**

In order to minimize positional effect and to guarantee the presence of all Pet1 regulatory elements, the RP23_165_D11 BAC clone comprising 170 kb upstream and 40 kb downstream the Pet1 gene locus has been used to drive the expression of Cre recombinase in a transgenic-based approach in the mouse. A homologous recombination strategy in Drosophila melanogaster was used to generate the Pet1210-Cre transgene (Figure S1). After pronuclear injection, Southern Blot analysis on genomic DNA using a probe designed against the Kana/Neo DNA sequence allowed the identification of four independent Pet1210-Cre founders, three of which showed germline transmission, namely founder-female 3 (FF3), founder-female 9 (FF9) and founder-male 3 (FM3). Pet1210-Cre founders were intercrossed to Actb::FLPe deleter mice [41] to remove the Kana/Neo resistance cassette to avoid possible transcriptional interference with the Pet1 promoter. Genomic DNA was assayed by PCR and sequencing to assess correct Flp-mediated excision of the FRT-flanked Kana/Neo cassette (not shown). Eventually, the three Pet1210-Cre founders were backcrossed to a C57BL/6j background for at least nine generations. The transgenic mice appeared to be morphologically normal, had a normal lifespan and were fertile, thus suggesting no consequences due to the passenger genes (i.e. Cdk5r2, Cryba2, Cdc108, Ihh and Nheg1) included in the Pet1210-Cre BAC construct (Figure S1).

In order to characterize the newly generated transgenic lines, we first tested the Cre somatic recombination efficiency by intercrossing Pet1210-Cre transgenic animals obtained from the 3 distinct founders to the ROSA26R conditional reporter line, in which β-galactosidase is constitutively expressed upon Cre-mediated recombination [42].

It is reported that in the mouse hindbrain Pet1 expression starts around 10.5 dpc in the rostral serotonergic domain, and about one day later in the caudal raphe nuclei [31,32]. X-gal analyses revealed that Cre-mediated recombination had occurred already in the hindbrain of 11.5 dpc Pet1210-Cre/ROSA26R mouse embryos showing two longitudinal dark blue stripes lateral to the floorplate defining the rostral serotonergic domain. Conversely, β-galactosidase staining in the medullary domain was barely detectable at this stage, reflecting the rostro-caudal temporal order in the generation of raphe serotonergic neurons (Figure 1a). At 12.5 dpc the analysis on sagittal sections and hindbrain flat-mount preparation from the three distinct founders showed that the transgene was expressed both in the anterior r1-r3-derived and in the posterior r3-r7-derived hindbrain regions, with the exception of the r1-derived territory, thus mirroring the endogenous expression of the Pet1 gene (Figure 1b and Figure S2 a–c). At P1, β-galactosidase-expressing neurons have migrated from their original position in the ventral region of the hindbrain and reached their final location in the brainstem, defining dorsal, medial and medullary clusters of serotonergic neurons (Figure 1c), in line with the morphogenetic movements of the developing serotonergic system occurred at this stage [43]. β-galactosidase
staining was confirmed in all serotonergic B1–B9 raphe nuclei of adult Pet1210-Cre/ROSA26R transgenic mice (Figure 1d–g, d'-g'), but not in ectopic districts as assessed by a detailed analysis on coronal sections throughout Pet1210-Cre/ROSA26R mouse brains (Figure 1h–i, h'-i', h''-i''), which also showed that the transgene expression nicely correlates to Pet1 endogenous expression (Figure S3), and to that of the specific marker of terminally differentiated 5-HT neurons such as Tph2.

Thus, the Pet1210-Cre transgenic mouse line Cre recombinase expression likely mirrors Pet1 spatio-temporal localization in the raphe nuclei during both foetal development and post-natal life. As the three founders showed similar Cre activity, we selected the FP9-derived mice, which showed the strongest β-galactosidase signal, to perform the detailed analysis described below. For this founder further genomics analysis was performed in order to assess the integrity of the BAC transgene and the transgene copy number integrated into the genome. Evidence that the BAC transgene was intact within the chromosomal DNA of Pet1210-Cre mice was deduced by the amplification of the 142 bp and 254 bp fragments corresponding to the 5'- and 3'-end, respectively, of the PI-SceI linearized BAC backbone (Figure S1 c). Furthermore, Southern blot analysis showed that the BAC transgene was integrated as a single copy into the genome of FF9-derived mice (Figure S1 d).

**Identification of a non-serotonergic Pet1**

We then intercrossed the Pet1210-Cre mice with the ROSA26YFP conditional reporter line [44], in which the YFP reporter gene is constitutively activated upon Cre expression, in order to assess the Cre-mediated recombination activity at a cellular level. Combined double immunohistochemistry experiments were performed using specific antibodies against YFP and 5-HT on sections from Pet1210-Cre/ROSA26YFP double transgenic mouse brains (Figure 2 a–e).

Results highlighted a discrepancy between YFP and 5-HT immunoreactivity within the raphe nuclei along the rostro-caudal extent of the hindbrain. In particular, while virtually all 5-HT-immunoreactive cells resulted positive for YFP (YFP+/5-HT+), several YFP-immunoreactive cells resulted to be devoid of serotonin (YFP+/5-HT−, Figure 2 a-a′″, b-b′″, c-c′″, d-d′″, e-e′″). In particular, the fraction of YFP+/5-HT− vs YFP+/5-HT+ cells displayed a rostral-to-caudal decreasing ratio, being sizable in the rostral median raphe (MR) B8–B9 nuclei (Figure 2 a-a′″, b-b′″), while in dorsal raphe (DR) B7 (Figure 2 c-c′″), B6 (Figure 2 d-d′″) and caudal B1–B3 nuclei (Figure 2 e-e′″) only few YFP+/5-HT− were present.

Thus, our results raised the question whether the unexpected presence of YFP+/5-HT− cells in the adult mouse brain could be due to an ectopic activity of the Cre recombinase or to a specific, novel expression domain of Pet1 gene in a non-serotonergic cell subpopulation. To answer this question, we performed combined double in situ hybridization experiments on adult wild-type mice using specific riboprobes for both Pet1 and Tph2, with the latter being expressed selectively in all terminally differentiated serotonergic neurons within the raphe nuclei (Figure 3 a–e). Consistently with immunohistochemistry data, few neurons expressing Pet1 but devoid of Tph2 were detected in dorsal and caudal raphe B6 (Figure 3 d-d″), B7 (Figure 3 c-c′″) and B1–B3 (Figure 3 e-e′″) nuclei whereas in B9 (Figure 3 a-a″) and B8 (Figure 3 b-b″″) raphe nuclei a substantial number of non-serotonergic neurons resulted positive for Pet1 expression. In order to quantify this observation, both Pet1+/Tph2− and Pet1+/Tph2+ neurons were counted in B9, B8, B7 and B6 rostral raphe nuclei and in the B1–B3 caudal cluster. Analysis showed that in B1–B3 group, in B6 and in B7 raphe nuclei the percentage of Pet1+/Tph2− neurons was 1.5%, 1.1% and 0.8%, respectively, while in B8 and in B9 nuclei it reached 17.6% and 25.5%, respectively (Figure 3f). Further, to address at the cellular level whether in our Pet1210-Cre transgenic mouse line the Cre recombinase promoted somatic recombination mirroring the Pet1 expression pattern, we used two distinct riboprobe combinations (i.e. Pet1 and YFP, or Tph2 and YFP), to perform double ISH on serial coronal sections of adult Pet1210-Cre/ROSA26YFP. Analyses of results confirmed that the reporter YFP is present in all Pet1−/− neurons as highlighted by co-expression of Pet1 and YFP (Figure 4 c-c″), and demonstrated that the Pet1 promoter is also active in non-serotonergic neurons as shown by the partially overlapping expression of Tph2 and YFP (Figure 4 b-b″), in line with the presence of a Pet1+/Tph2− neuronal population in the raphe system (Figure 4 a-a″). Moreover, as NBT/BCIP deep purple chromogenic precipitate may quench the fluorescence generated by the HNPP/Fast Red substrate, masking the Tph2 signal in Pet1 positive neurons, we repeated double ISH on coronal sections at the level of B5 raphe nucleus swapping the detection methods for Pet1 (HNPP/Fast Red) and Tph2 (NBT/BCIP) riboprobe as an additional control. We intentionally let the staining with NBT/BCIP substrate proceed until saturation was reached, so that Tph2 expressing neurons showed a very dark blue signal likely masking any underlying fluorescence. In spite of that, Pet1−/− positive neurons identified by alkaline-phosphatase activity using HNPP/Fast Red substrate were still clearly visible (Figure 4 d-d″). Together, these data indicate that Pet1210-Cre mouse line promotes Cre recombinase expression in all serotonergic neurons and identifies for the first time a novel, non-serotonergic raphe neuronal population expressing Pet1.

**Pet1 gene is expressed in developing pancreas and kidney**

Analysis of the spatio-temporal domain of the Cre recombinase activity in the Pet1210-Cre/ROSA26R mouse embryos from all the three founders at both 11.5 dpc and 13.5 dpc revealed the presence of β-galactosidase staining in additional potential Pet1 expression domains outside the CNS unprecedently described (Figure 5). At 11.5 dpc Cre-mediated recombination was identified in the anlage of the pancreas and in the ureteric bud of the developing kidney, which at this stage forms a small branch at the level of the hindlimbs at the terminal extremity of tubular structures on each side of the caudal abdominal region (Figure 5 a–c). At 13.5 dpc, X-gal staining performed on parasagittal sections of Pet1210-Cre/ROSA26R embryos corroborated the presence of Cre recombinase activity in the developing pancreas and in the branching ureteric bud of the kidney (Figure 5 d–i). Additionally, X-gal staining performed on whole organs or sections obtained from Pet1210-Cre/ROSA26R pups at birth or adult animals, showed that while both pancreas and kidneys confirmed the presence of X-gal staining, no reporter expression was evident in all the other Pet1210-Cre/ROSA26R organs analysed (Figure S4).

The presence of β-galactosidase activity in both pancreas and kidneys of mice derived from all the Pet1210-Cre founders prompted us to hypothesize that the expression of the conditional reporter was due to specific, previously unreported Pet1 expression domains, rather than an ectopic activation of Cre recombinase. To address this hypothesis we performed ISH experiments on pancreas and kidneys from 15.5 dpc Pet1210-Cre/ROSA26YFP embryos, using specific riboprobes for Pet1 and YFP. Results indicated that YFP and Pet1 are expressed in the same pancreatic...
domains, nicely correlating with the pancreatic islet cell marker Nkx2.2 (Figure S5 a–c). Moreover, despite in the hindbrain the expression of Pet1 normally preludes to the acquisition of a serotonergic phenotype, we could not detect either Tph2 or Tph1 expression in the developing pancreas (Figure S5 d–e).

To assess the presence of Pet1 mRNA in the kidney, due to its low expression level, we performed radioactive ISH on coronal sections of 12.5 dpc Pet1210-Cre/ROSA26R embryos highlighting the presence of the reporter in both the rostral (arrows) and caudal (arrowheads) clusters of developing serotonergic neurons. (c) In P1 Pet1210-Cre/ROSA26R brains X-gal staining highlights serotonergic neurons migrated towards their terminal locations within the rhombencephalon. (d–g, d’–g’). Representative coronal sections throughout the antero-posterior extent of the raphe of an adult (P45) Pet1210-Cre/ROSA26R brain showing Cre-mediated recombination specifically occurred in all serotonergic nuclei, namely B8–B9 (d, d’), B7 (e, e’), B5–B6 (f, f’) and B1–B3 (g, g’). (h–h”, i–i”) no β-galactosidase staining is detectable in anterior brain regions such as cortex (h, h”), hippocampus (h, h”), substantia nigra (i, i”) and thalamus (i, i”). Scale bar: 1 mm (a, d–i), 900 μm (c), 600 μm (b), 300 μm (d’–i’, h”, i”).

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Figure 1. Pet1210-Cre mouse line drives Cre recombinase activity in the serotonergic system. (a) Dorsal view of a cleared X-gal stained Pet1210-Cre/ROSA26R embryo at 11.5 dpc showing that Cre-mediated somatic recombination has occurred in the rostral raphe (arrow). (b) Sagittal section of 12.5 dpc Pet1210-Cre/ROSA26R embryo highlighting the presence of the reporter in both the rostral (arrows) and caudal (arrowheads) clusters of developing serotonergic neurones. (c) In P1 Pet1210-Cre/ROSA26R brains X-gal staining highlights serotonergic neurones migrated towards their terminal locations within the rhombencephalon. (d–g, d’–g’) Representative coronal sections throughout the antero-posterior extent of the raphe of an adult (P45) Pet1210-Cre/ROSA26R brain showing Cre-mediated recombination specifically occurred in all serotonergic nuclei, namely B8–B9 (d, d’), B7 (e, e’), B5–B6 (f, f’) and B1–B3 (g, g’). (h–h”, i–i”) no β-galactosidase staining is detectable in anterior brain regions such as cortex (h, h”), hippocampus (h, h”), substantia nigra (i, i”) and thalamus (i, i”). Scale bar: 1 mm (a, d–i), 900 μm (c), 600 μm (b), 300 μm (d’–i’, h”, i”).

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reporter became detectable as early as 9.5 dpc in scattered cells along the developing nephric duct (Figure 6 g-g'). At 10.5 dpc X-gal staining was present in the nephric duct showing an increasing intensity toward its caudal end (Figure 6 h-h'). At 13.5 dpc β-galactosidase staining nicely highlighted that ureteric bud underwent already through different rounds of branching forming a tree-like structure (Figure 6 i-i'). Finally, in P1 mice X-gal staining was present in cells belonging to the collecting duct epithelium (Figure 6 j-j'). Importantly, no X-gal staining was observed within the metanephric mesenchyme, thus suggesting that in Pet1210-Cre transgenic mouse line Cre-mediated recombination occurred specifically within the ureteric bud precursors. No Cre recombinase activity was detected in the adrenal gland of Pet1210-Cre/ROSA26R mice (Figure S4 c, i-i') as expected in agreement with Fyodorov and collaborators (1998) [45].

In order to confirm that Pet1 expression is localized within the ureteric bud derivatives, we performed immunohistochemistry experiments on Pet1210-Cre/ROSA26YFP whole mount kidneys at 13.5 dpc using antibodies against YFP and the specific marker for ureteric bud calbindin-D28K [46]. Results showed that cells expressing YFP were also immunoreactive for calbindin-D28K, which stains both tips and stalks of ureteric bud branches (Figure 6 k–n, k'-n'). In contrast, not all calbindin-D-28K+ cells...
expressed YFP, indicating that Cre-mediated recombination in the kidney occurred in a subset of calbindin-D28K expressing cells. Thus, the characterization of the Pet1210-Cre transgenic line allowed the identification of novel expression domains outside the CNS where Cre activity faithfully recapitulates Pet1 expression.

Discussion

In the present study we reported the generation and the comprehensive characterization of the Pet1210-Cre transgenic mouse line, in which a BAC-derived 210 kb genomic fragment of Pet1 gene locus was used to drive Cre recombinase expression. To date, it represents the largest Pet1 genomic region used to drive Pet1-like transgene expression. We observed that in Pet1210-Cre transgenic mouse line the 210 kb region driving Cre recombinase expression was able to recapitulate the timing of the endogenous Pet1 expression during serotonergic system development, as well as the spatial distribution within B1–B9 raphe nuclei. This result, together with the absence of reporter expression in extra-raphe domains in the brain, indicated a reliable, Pet1-driven, spatial and temporal control of Cre expression in the Pet1210-Cre mouse line. These results are in line with the peculiarities of BAC-based transgenesis, that limits positional effects and guarantees the presence of long-range acting regulatory elements as well, likely promoting transgene expression in an endogenous-like manner independently of the integration site [34,36–38,47]. Furthermore, despite the expression of Pet1 in serotonergic neurons observed in our Pet1210-Cre transgenic mice is consistent with data previously obtained with other Pet1-based transgenic mouse lines [16,22,48,49], in the present study we demonstrated that Pet1 expression in the raphe is present in a wider domain than previously described, as assessed by double IHC characterization of Pet1210-Cre transgenic line and by ISH experiments on wild-type animals with different combination of Pet1 and Tph2 probe staining. Interestingly, we observed that within the raphe system the non-serotonergic Pet1-positive neurons are unevenly rather than homogeneously distributed along the raphe, with a substan-

Figure 3. Pet1 gene is expressed in a non-serotonergic raphe cell population in adult mice. (a–e′′) Low (a, b, c, d, e) and high (a′-a′′, b′-b′′, c′-c′′, d′-d′′, e′-e′′) magnification images of double ISH performed on coronal sections obtained from adult wild-type mice at the level of B9 (a-a′′), B8 (b-b′′), B7 (c-c′′), B6 (d-d′′), and B1–B3 (e-e′′) raphe nuclei. In each picture, Pet1 expression is highlighted by a dark blue staining (a-a′, b-b′, c-c′, d-d′, e-e′), while Tph2 gene expression is visualized as a red precipitate (a-a′, b-b′, c-c′, d-d′, e-e′), or as a red fluorescence (a′-a′′, b′-b′′, c′-c′′, d′-d′′, e′-e′′). Boxed areas indicate the regions shown in higher magnification images. In all the raphe nuclei two distinct populations of neurons expressing either both Pet1 and Tph2 (arrowheads), or only Pet1 (arrows) are present. (f) Ratio of Pet1+/Tph2+ vs Pet1+/Tph2− neuronal population in distinct raphe nuclei of adult wild-type mice reported as percentage. Histogram shows that Pet1-positive non-serotonergic neurons are significantly represented in rostral B8 and B9 as compared to more posterior nuclei. Data are presented as mean ± SEM. Scale bar: 100 μm (a–e), 25 μm (a′-a′′, b′-b′′, c′-c′′, d′-d′′, e′-e′′).

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It has been well established that the majority of neurons populating the raphe are not serotonergic, even though the term raphe is often used as a synonym of serotonergic [50–52]. Both dorsal raphe and median raphe nuclei, which together provide the main serotonergic innervation to the brain, are also composed by a heterogeneous population of neurons expressing different transmitter substances, such as glutamate [53], GABA [54], or peptides (e.g. corticotropin-releasing factor and substance P) [29]. The composition and the distribution of non-serotonergic neurons, as well as serotonergic neurons, show distinct pattern in DR and MR nuclei, often reflecting different target brain regions or subregions of the same brain structure such as amygdala, hippocampus and medial septum among the others [28,50,55–57]. Retrograde tracing studies have demonstrated that a large population of non-serotonergic neurons of the MR expresses the vesicular glutamate transporter VGLUT3, while in DR serotonin and VGLUT3 are often overlapping [57,58]. The evidence that other transmitter substances may be co-released with serotonin hampers the study of non-serotonergic populations of the raphe and, therefore, the possibility to better characterize the Pet1+/Tph2− neuronal population. Although beyond the intent of the present study, multiple labelling studies would be of great interest in future research in order to identify the molecular and neurotransmission characteristics of Pet1+/Tph2− cells. In this regard, intersectional and subtractive strategies have provided powerful tools to map cell subtypes with great precision [22,59,60]. Jensen and collaborators have shown that serotonergic neurons can be genetically defined on the basis of their rhombomeric origin more than their final localization in adult serotonergic nuclei. In particular, it has been reported that while DR (B7, B6, B4) neurons derive uniquely from r1, median nuclei (B9, B8, B4) derive from r1–r3 precursors [22]. Thus, in line with this study, it can be hypothesized that the Pet1-

Figure 4. Pet1210-Cre transgene specifically identify Pet1-expressing, non-serotonergic raphe neurons. Double ISH performed on serial coronal sections of adult Pet1210-Cre/ROSA26-YFP mouse brains at the level of B8 raphe nucleus using combination of Pet1/Tph2 (a-a’), YFP/Tph2 (b-b’), YFP/Pet1 (c-c’) and Tph2/Pet1 (d-d’) riboprobes. In each combination the former probe is highlighted using the NBT/BCIP substrate, while the latter using the Fast Red chromogen. Boxed areas are shown at higher magnification in brightfield (a’, b’, c’, d’) or fluorescence (a’’, b’’, c’’, d’’). While all Pet1-positive cells also express YFP (arrowheads in c’-c’’), the presence of a Pet1-positive, non-serotonergic cell population was confirmed with all the other probe combinations (arrows in a’-a’’, b’-b’’, c’-c’’). Scale bar: 100 μm (a-d), 25 μm (a’-a’’, b’-b’’, c’-c’’).
positive non-serotonergic neurons present in raphe nuclei likely arise from a heterogeneous population of precursors deriving from r1 to r3. The combination of suitable transgenic and conditional reporter mouse lines in an intersectional approach may represent a valuable tool to dissect the developmental origin and the nature of Pet1-positive, non 5-HT neurons. The possibility to shed light on the molecular identity of Pet1+/Tph2- neurons will potentially impact on those studies in which the Pet1 regulatory region has been used to drive Cre recombinase expression, in order to specifically target serotonergic neurons. Indeed, in light of our results, the non-serotonergic expression might have contributed to the observed phenotypes resulting from Pet1-driven Cre-mediated somatic recombination in conditional knockouts.

The use of Pet1210-Cre transgenic mouse line allowed the tracing of Pet1 expressing cell progeny in the developing pancreas.

Indeed, our data are consistent with the report by Ohta and collaborators, who have shown, using the ePet-Cre transgenic mouse line [61], that Pet1 is expressed in both developing and adult pancreas, peaking at E14.5 and co-localizing with endocrine-specific markers such as glucagon and insulin or specific transcription factors as Nkx2.2. In the same study, the analysis of Pet1 mutant animals showed a reduction of the expression of insulin genes, resulting in glucose clearance and insulin secretion defects. Interestingly, Pet1 does not directly promote Tph2 expression in the pancreas, suggesting that the Pet1-mediated regulation of either pancreatic β-cell or serotonergic neuron development proceeds via distinct genetic cascades [61]. More importantly, to our knowledge the present work provides the first evidence of Pet1 expression in kidney. Lack of previous reports showing Pet1 expression in the ureteric bud derivatives may be

Figure 5. Pet1 drives Cre-mediated recombination in the developing pancreas and kidney. X-gal staining performed on Pet1210-Cre/ROSA26R whole mount 11.5 dpc embryos (a–c) and on sagittal sections at the level of the developing pancreas (d–f) and kidney (g–i) of 13.5 dpc embryos obtained from FF3 (a, d, g), FF9 (b, e, h) and FM3 (c, f, i) founder mouse lines. At 11.5 dpc a clear expression of the reporter is present at comparable levels in the three founder lines in both the developing pancreas (arrows) and kidneys (arrowheads), while it is still barely detectable in the hindbrain (asterisks). At 13.5 dpc X-gal staining highlights cells in the developing pancreas (d–f) and in the branching ureteric bud of the kidney (g–i) where Cre-mediated somatic recombination has occurred. Scale bar: 600 μm (a–c), 150 μm (g–i), 100 μm (d–f). doi:10.1371/journal.pone.0104318.g005
Figure 6. Pet1 is expressed in the ureteric bud-derived tissues during kidney development. (a–f) Radioactive ISH performed on coronal section of 10.5 dpc (a–b), 11.5 dpc (c–d) and 12.5 dpc (e–f) Pet1210-Cre/ROSA26YFP (a–d) and wild-type (e–f) embryos. Results show presence of Pet1 mRNA expression in the nephric duct (arrowheads) already at 10.5 dpc (a), mirroring YFP reporter expression (b). At 11.5 dpc both Pet1 and YFP expression is highlighted in the two forming ureteric buds (c–d, arrowheads), while Pet1 but not the reporter expression is detectable in 12.5 dpc wild-type embryos (e–f), confirming the specificity of Pet1 expression in the developing kidney. (g–j') X-gal staining performed on whole-mount 9.5 dpc (g, g'), 10.5 dpc (h, h'), 13.5 dpc (i, i'). (j, j') Pet1210-Cre/ROSA26R mouse embryos and on coronal sections of a P1 Pet1210-Cre/ROSA26YFP mouse kidney 

Our findings on the spatio-temporal expression domain of Pet1 go beyond the results obtained using the previously generated ePet1-Cre transgenic mouse line, in which presence of Pet1 in the kidney was excluded [62]. This discrepancy might be due to a number of factors, with the most likely being that the regulatory elements necessary to drive a Pet1-like expression in the kidney are likely included in the 210 kb Pet1 entire regulatory region of the Pet1210Cre transgene, and not present in the 40 kb region used to generate the ePet1-Cre transgene [62]. In good agreement with our observation that Pet1 is expressed in mammalian kidney, microarray analysis performed on mouse and rat transcriptomes indicated that Pet1 transcript was enriched in the ureteric bud as compared to other cell types in the developing kidney such as the metanephric mesenchyme [63]. Nevertheless, although Pet1 expression has been shown in the adrenal gland by Fyodorov and collaborators [45], Cre recombinase activity in this district was not detected either by Scott and collaborators [16], or from our analysis, using ePet-Cre and ePet-YFP, or Pet1210Cre transgenic mouse lines, respectively. The presence of an intact recombinant BAC in Pet1210Cre mice suggests that the specific enhancer driving expression of Pet1 in the adrenal gland may be a long-range acting regulatory element, which is not likely present in the 210 kb genomic region contained in the RP23_165_D11 BAC clone.

Interestingly, as already observed for pancreas development [61], also in the kidney Pet1 is co-expressed with other transcription factors shared with the serotonergic differentiation pathway. It has been reported that LMX1B mutation causes nephropathies and, often, renal failure in human patients affected by Nail-Patella syndrome [64,65]. Mouse Lmx1b is expressed in podocytes of the kidney, and its genetic ablation results in kidney defects resembling those observed in Nail-Patella syndrome patients [66,67]. On the other hand, the functional role of Pet1.
in the kidney has not been yet characterized. It is known that chromosomal translocations in which Ewing's sarcoma (EWS) gene is fused to a variety of transcription factors, including human FEV, can lead to the onset of different subsets of Ewing tumours [68,69], with some of them occasionally occurring in the kidney [70].

On the whole, the use of Pet1210-Cre transgenic mouse line allowed the identification of novel Pet1 expression domains both in the hindbrain and in the developing kidney. As the expression of the reporter is permanently induced following recombination, this line will allow long-term analysis of Pet1-expressing cell types, both in non-serotonergic neurons as well as in non-serotonergic cell populations of the raphe. Moreover, this mouse line constitutes a valuable model for studying molecular mechanisms of renal and pancreatic development and function by induction or silencing of specific genes in epithelial ureteric bud-derivatives and islet cells, respectively. Finally, given the expression of Pet1 in extra-serotonergic neurons within the raphe system, when using Pet1-driving Cre transgenic mouse lines in conditional gene targeting approaches or fate mapping studies aimed to target serotonergic cells, it should be taken into great consideration that the phenotype observed might arise from a combined, rather than serotonergic-specific, somatic recombination.

Supporting Information

Figure S1 Pet1210-Cre allele generation. Diagram showing the wild-type Pet1 genomic locus contained within the RP23_165D11 BAC clone (a), the targeting vector for the homologous recombination in E. coli DY380 cells and the resulting Pet1210-Cre transgenic allele before and following Flp-mediated recombination. (b) Southern Blot analysis performed on genomic DNA obtained from Pet1210-Cre founders by BamHI digestion and hybridization with a probe against Kana/Neo cassette. Among the founders analysed, 4 presented the expected band at 2.6 kb. Founder-male 3 (FM3, lane 1), founder-female 3 (FF3, lane 7) and founder-female 9 (FF9, lane 10) showed germline transmission. (c) A 142 bp fragment at the 5’-end and a 234 bp fragment at the 3’-end of the transgene were amplified from the Pet1210-Cre FF9 founder after NotI digestion and hybridization with a probe against Kana/Neo cassette. From the Southern Blot analysis performed on genomic DNA obtained from Pet1210-Cre FF9 founder after NotI digestion and hybridization with a probe capable to discriminate wt Pet1 locus vs Pet1210-Cre transgene. A clear lower hybridization intensity of the Pet1210-Cre transgene (4.2 kb) as compared to the Pet1 wt allele (3.1 kb) confirms the presence of a single copy of the transgene. (d) Southern Blot analysis performed on genomic DNA obtained from Pet1210-Cre FF9 founder after NotI digestion and hybridization with a probe capable to discriminate wt Pet1 locus vs Pet1210-Cre transgene. Moreover, this mouse line constitutes a valuable model for studying molecular mechanisms of renal and pancreatic development and function by induction or silencing of specific genes in epithelial ureteric bud-derivatives and islet cells, respectively. Finally, given the expression of Pet1 in extra-serotonergic neurons within the raphe system, when using Pet1-driving Cre transgenic mouse lines in conditional gene targeting approaches or fate mapping studies aimed to target serotonergic cells, it should be taken into great consideration that the phenotype observed might arise from a combined, rather than serotonergic-specific, somatic recombination.

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Figure S3 Pet1210-Cre somatic recombination mirrors Pet1 spatio-temporal expression in the serotonergic system. Representative sagittal 12.5 dpc, and coronal 15.5 dpc, P10 and P30 Pet1210-Cre/Rosa26R double transgenic (a, d, g, j) or wild-type (b–c, e–f, h–i, k–l) brain sections stained with X-gal chromogenic reaction or hybridized with a Pet1 (b, e, h, k) or a Tph2 (c, f, i, l) riboprobe, respectively. Note that at all the stages analysed β-galactosidase activity parallels with both Pet1 and Tph2 expression. Scale bar: 1 mm (a–c), 200 μm (d–i), 150 μm (j–l).

Figure S4 Cre-mediated recombination outside the raphe in Pet1210-Cre mouse line selectively occurs in pancreas and kidney. X-gal staining performed on whole-mount tissues from Pet1210-Cre/Rosa26R (a, b, c, d, e, f, g, h, i, j, k, l) and Rosa26R (a’, b’, c’, d’, e’, f’, g’, h’, i’, j’, k’) animals at P 0.5 (a–f*) or adult (g–l*), showing the early postnatal and terminal distribution of Pet1-expressing cell progeny both in the brain and in peripheral organs. Cre-mediated recombination occurs specifically in hindbrain (a), pancreas (b) and kidneys (c) of P 0.5 double transgenic pups, and it is confined to the mature raphe system (g, g’), pancreatic beta cells (h, h’) and renal UB-derived collecting ducts and ureter (i, i’) in adults. Evidence of Cre-mediated recombination was undetectable in whole-mount specimens and on sections of adrenal glands (arrow in c, insets in i and i’, respectively). No reporter expression is present either in heart (d, j), spleen (e, k) or liver (f, l). The expression pattern of Pet1210-Cre/Rosa26R, or in organs from Rosa26R mice (a’, b’, c’, d’, e’, f’, g’, h’, i’, j’, k’, l’). Scale bar: 1.7 mm (g-g’, i-i’, j-j’, k-k’, l-l’), 1.5 mm (a-a’, b-b’, c-c’, f-f’, inset in i-i’), 750 μm (d-d’, e-e’, h-h’).

Figure S5 Pet1 expression in the developing pancreas correlates with the reporter distribution in Pet1210-Cre/Rosa26YFP mouse line. Images of serial coronal sections at the level of pancreas of Pet1210-Cre/Rosa26YFP 15.5 dpc embryos hybridized with Pet1 (a), VFP (b), Nkx2.2 (c), Tph1 (d) and Tph2 (e) riboprobes. Pet1 expression correlates with the expression of both VFP and Nkx2.2, while neither Tph2 nor Tph1 expression is detected in the pancreas at this stage. Scale bar: 300 μm (a–e).

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Author Contributions

Conceived and designed the experiments: BP M. Pasqualetti. Performed the experiments: BP SM GP M. Pasqualetti M. Pratelli. Analyzed the data: BP SM M. Pasqualetti. Contributed to the writing of the manuscript: BP M. Pasqualetti.
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