Genetic Variants in the STMN1 Transcriptional Regulatory Region Affect Promoter Activity and Fear Behavior in English Springer Spaniels

Xiaolin Ding¹*, Jin Hu¹, Hanying Zhang², Yinxue Xu¹

¹ Department of Animal Genetics, Breeding and Reproduction, College of Animal Science and Technology, Nanjing Agricultural University, Nanjing, Jiangsu Province, People’s Republic of China, ² Pharmacology Department, R&D center, Nanjing Sanhome Pharmaceutical Co. LTD, Nanjing, Jiangsu Province, People’s Republic of China

☯ These authors contributed equally to this work.
* xuyinxue@njau.edu.cn

Abstract

Stathmin 1 (STMN1) is a neuronal growth-associated protein that is involved in microtubule dynamics and plays an important role in synaptic outgrowth and plasticity. Given that STMN1 affects fear behavior, we hypothesized that genetic variations in the STMN1 transcriptional regulatory region affect gene transcription activity and control fear behavior. In this study, two single nucleotide polymorphisms (SNPs), g. -327 A > G and g. -125 C > T, were identified in 317 English Springer Spaniels. A bioinformatics analysis revealed that both were loci located in the canine STMN1 putative promoter region and affected transcription factor binding. A statistical analysis revealed that the TT genotype at g.-125 C > T produced a significantly greater fear level than that of the CC genotype (P < 0.05). Furthermore, the H4H4 (GTGT) haplotype combination was significantly associated with canine fear behavior (P < 0.01). Using serially truncated constructs of the STMN1 promoters and the luciferase reporter, we found that a 395 bp (−312 nt to +83 nt) fragment constituted the core promoter region. The luciferase assay also revealed that the H4 (GT) haplotype promoter had higher activity than that of other haplotypes. Overall, our results suggest that the two SNPs in the canine STMN1 promoter region could affect canine fear behavior by altering STMN1 transcriptional activity.

Introduction

Police dogs play a unique role in preventing and cracking down on crime [1]. In the Nanjing Police Dog Institute of the Ministry of Public Security of the People’s Republic of China, the training records of English Springer Spaniels show that many puppies are unable to be trained into working dogs because they are fearful. Fear can be affected by genetic, environmental...
As an emotional state, fear can protect the dog by defensive behavior or escape once it perceived danger [5, 6]. Fear can be triggered by various stimuli, such as novel or intense stimuli, special evolutionary danger, social interactions with conspecifics, and conditioned stimuli [7]. Fear-based behavioral responses include aggressive behavior [8, 9], avoidance responses [10], flight [11], withdrawal [12], and immobility (freezing or crouching) [13, 14]. As fearful subjective states cannot be directly measured by self-report in dogs, the experience of fear is based on proxy measures of behavioral responses. Fearful behavior in dogs can be assessed by objective behavioral testing or subjective questionnaire surveys. In general, few differences are found between behavioral tests and questionnaire surveys in the consistency of assessing the fearfulness of a dog [15–17]. The genetic background of fear is polygenic and most likely involves genes associated with different cellular processes and pathways, but the specifics remain unclear [18]. Thus, further studies to identify specific genes involved in the formation and expression of fear are needed.

The microtubule destabilizers Stathmin family, includes STMN1, STMN2 (SCG10: superior cervical ganglion-10 protein), STMN3 (SCG10-like protein) and STMN4 (stathmin-like protein B3) [19–22]. They have a common tubulin binding site which can destabilize MT and sequester tubulin heterodimer. STMN1 is widely distributed and localized in the cytosol [23], whereas STMN2, STMN3 and STMN4 are neuron-specific and located on membranes of developing (STMN2 and STMN3) and mature (STMN4) nerve cells [19–22]. STMN1 expresses highly in thalamus, cortex and lateral amygdala and regulates MT dynamics, synaptic growth and plasticity [24–26].

Several studies have reported that STMN1 is closely related to fear level in humans and animals. Two single nucleotide polymorphisms (SNPs) (rs182455 and rs213641) in the transcriptional control region of STMN1, were found to affect fear and anxiety responses in humans [27]. The results of a subsequent study showed that the rs182455 C-allele alters the cognitive-affective processing of healthy people and could affect fear processing [28]. In mice, knocking out STMN1 led to abnormal spike-timing-dependent long-term potentiation and defective memory on fear conditioning [29–31].

However, genetic variations in the STMN1 transcriptional regulatory region have not yet been reported in dogs. Moreover, the distribution, frequency, and function of the STMN1 variations are unclear, and the regulation of STMN1 transcriptional activity by the promoter region remains to be revealed. Therefore, the present study investigated potentially functional genetic variations in the STMN1 promoter and explored whether these genetic variations could affect gene transcriptional activity and, consequently, fear behavior.

Materials and Methods

Sample collection

A total of 317 English Springer Spaniels from the Nanjing Police Dog Institute of the Ministry of Public Security were employed in this study (Nanjing, Jiangsu Province, China). The ages of these dogs ranged from 6 to 10 months with 142 males and 175 females. Blood samples (2 mL) were collected from forelimb vein by a veterinarian and stored at −20°C for DNA extraction. Heart, liver, spleen, lung, kidney, pancreas, muscle, medial prefrontal cortex (mPFC), midbrain, cerebellum, brainstem, amygdala, hippocampus, hypothalamus, hypophysis, spinal cord, medulla oblongata, and olfactory bulb tissues were collected from 15 dogs, within 30 min of euthanasia by overdose of pentobarbital, frozen and stored in liquid nitrogen until total RNA extraction. All experimental procedures and sample collections were performed according to the Regulations for the Administration of Affairs Concerning Experimental Animals (Ministry of Science and Technology, China; revised in June 2004) and approved by the Ethics
Committee of Nanjing Agricultural University. All efforts were made to minimize the number of animals used and their suffering.

Fear behavioral test

The fear level test consisted of five separate subtests: the affability and handling test [17], floor test, gunshot test [32], umbrella test, and aluminum food bowl test [17, 33]. The tests were administered to 317 dogs, and were conducted outdoors in a specific order. All dogs were scored from 1 to 5 according to their fear reaction across all subtests, where 1 = no fear, 2 = slight fear, 3 = obvious fear, 4 = very frightened, and 5 = terrified. The results of behavioral tests are presented in S1 Table.

RNA preparation and STMN1 gene expression in different tissues

Total RNA was extracted from heart, liver, spleen, lung, pancreas, muscle, mPFC, midbrain, cerebellum, brainstem, amygdala, hippocampus, hypothalamus, hypophysis, spinal cord, medulla oblongata, and olfactory bulb tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) as previous described [34]. RNA purity was determined by the ratio of OD value at 260/280 nm (1.8–2.0). Total RNA was reversely transcribed in a 20 μL reaction mixture at 25°C for 10 min, 42°C for 30 min, and 85°C for 5 min with 5× QRT SuperMix (Vazyme, Nanjing, China). The primer pair sequences for the target genes (Table 1) were generated by Primer Premier 5 software. Real time quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed in a 20 μL system including 2 μL cDNA (50–100 ng/μL), 10 μL AceQ™ qPCR SYBR Green Master Mix (TransGen, Beijing, China), 0.4 μL ROX Reference Dye II, 0.8 μL (10 μM) of each primer, and 6 μL RNase-free water. PCR program is the same as previously described [35]. The qRT-PCR data were analyzed using the $2^{-ΔΔCt}$ method for mRNA quantification.

DNA preparation and screening for genetic variants in the promoter

Genomic DNA was extracted from blood samples using Proteinase K digestion, phenol chloroform extraction, and ethanol precipitation. The 317 total dogs were classified into two groups (n = 12) with high- or low-fear levels respectively. Two DNA pools for the high- and low-fear groups were prepared by mixing equal amounts of genomic DNA from 12 dogs. The transcription start site was marked as +1. The 5′-flanking transcriptional regulatory region (−2,034 nt to

| Table 1. Primers used in this study. |
|-----------------------------------|
| Primer | Length (bp) | Primer sequence (5′-3′) | Tm (°C) |
|--------|-------------|------------------------|--------|
| P-F1   | 2,387       | GGGGTTACCATGGTGGGAGGTTGTTG | 61     |
| P-F2   | 1,696       | GGGGTACCCGTTGGCAAGCAGTTG  | 58     |
| P-F3   | 1,293       | GGGGTTAAGCTGAGAAGCAGAGGAGG | 61     |
| P-F4   | 980         | GGGGTACCAGAAGGCTAGCAGTTG  | 58     |
| P-F5   | 675         | GGGGTACCGAAGCAGCTAGCAGTTG | 58     |
| P-F6   | 395         | GGGGTACCTGTGGGAGCAGTTG    | 58     |
| P-R    | -----       | CCGCTCGAGCAACAAATGAGCAGGGGAC | ----- |
| F1/R1  | 427         | TCTCATGGCCACAGATAG/GCTTGTGGCTTGGTTGTGG | 56     |
| F2/R2  | 405         | GGACTAGGCAATCTAAAC/GGACTAGGCAATCTAAAC | 56     |
| F3/R3  | 207         | ATCCGCCAAGCTTGCCTTGCCTTTAG/TCTTGGGGAATCCTCACTTGC | 60     |
| β-actin-F/R | 121 | GGGCCAGAAGGAAATCTTGCAGTAGTCGAGCAC | 60     |

doi:10.1371/journal.pone.0158756.t001
Genotyping of the polymorphisms

The sequencing results disclosed two genetic variations (g. -327 A > G and g. -125 C > T) in the canine STMN1 promoter. The g. -327 A > G and g. -125 C > T genotypes were examined using the polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP) technique. The F1/R1 and F2/R2 primers (Table 1) were designed to amplify the 427 bp and 405 bp PCR products harboring g. -327 A > G and g. -125 C > T, respectively. PCR amplifications system contains 100 ng DNA template, 10 μL 2× Taq Premix, 0.5 μL of each primer (10 μM), and double distilled H2O up to 20 μL. The cycling protocol is the same as previously described [36]. Aliquots of 10 μL of the PCR products were mixed with 10 μL denatured solution, incubated at 98°C for 10 min, and chilled on ice. The denatured DNA was subjected to 8% polyacrylamide gel electrophoresis in 1× TBE buffer and separated at a constant 160 v for 16 h. The gel was stained with 0.1% silver nitrate and visualized with 2% NaOH solution (containing 0.1% formaldehyde) [37]. The PCR products from the different PCR-SSCP genotypes were sequenced.

Cloning and construction of the STMN1 reporter plasmids

To evaluate the promoter activity of the STMN1 transcriptional regulatory region, we conducted serial truncations of the STMN1 promoter fragment, ranging from −2,304 nt to +83 nt, to analyze reporter construct activity. The six STMN1 primer pairs are listed in Table 1. The forward and reverse primers contained the Kpn I and Xho I restriction sites, respectively. The PCR products were purified, and the target DNA fragments were digested with the Kpn I and Xho I restriction enzymes and cloned into multiple cloning sites (Kpn I and Xho I) of the pGL3-basic vector (Promega, Madison, WI, USA). The recombinant plasmids were confirmed by sequencing and denoted pGL3-F1, -F2, -F3, F4, F5, and F6, respectively. To compare the transcriptional activities of the STMN1 promoters between various haplotypes, the regulatory regions from −592 nt to +83 nt containing the H1(AC), H2(AT), H3(GC), and H4(GT) haplotypes were amplified with the P-F5/R primer pair (Table 1). The amplified products were digested with the Kpn I and Xho I restriction enzymes and subsequently cloned into multiple cloning sites of the pGL3-basic vector. The constructs were denoted pGL3- H1, -H2, -H3, and -H4, respectively.

Luciferase assay for promoter activity

Before transfection, 293T cells were seeded in 12-well plates. When cells had grown to 70–80% confluence, 0.05 μg pRL-TK vector (Promega) and 1 μg pGL3 vector were co-transfected with 3 μL Lipofectamine™ 2000 reagent (Invitrogen) following the manufacturer’s instructions. The pRL-TK vector is an internal control to correct for differences in transfection and harvesting efficiency. The luciferase assay was conducted as the manufacturer instructed (Promega). Firefly and Renilla luciferase activities were measured with a luminometer (Modulus; Turner Biosystems, Sunnyvale, CA, USA). Promoter activity is reported in relative light units and normalized against that of the empty pGL3-basic vector. All transfections were performed in triplicate and repeated at least three times in independent experiments.

Statistical analysis

Genotype frequencies, allelic frequencies, effective number of alleles, heterozygosities, polymorphism information content (PIC), and the chi-square test of goodness-of-fit for the Hardy-
Weinberg equilibrium law were calculated using PowerMarker V3.25 [38, 39]. Statistical comparisons were made by one-way ANOVA followed by Duncan’s multiple comparisons test using SPSS for Windows software (ver. 18.0; SPSS Inc. Chicago, IL, USA). P-values < 0.05 were considered significant. All data are expressed as means ± standard error.

Results

STMN1 expression analysis and correlation with fear behavior in English Springer Spaniels

Real-time PCR was performed to examine STMN1 expression in 18 tissues (heart, liver, spleen, lung, kidney, pancreas, muscle, mPFC, midbrain, cerebellum, brainstem, amygdala, hippocampus, hypothalamus, hypophysis, spinal cord, medulla oblongata, and olfactory bulb) of the dogs. High levels of STMN1 transcripts were detected mainly in brain tissues (amygdala, brainstem, mPFC, and hippocampus), and low levels were detected in heart, liver, spleen, kidney, pancreas, and muscle (Fig 1A). Four brain tissues were selected to compare STMN1 transcription levels between the high- and low-fear level dog groups. The RT-qPCR assay revealed that the high-fear level group expressed higher STMN1 mRNA levels than those of the low-fear level group (P < 0.05) in the amygdala, mPFC, and hippocampus (Fig 1B), but not in the brainstem. A significant correlation was detected between STMN1 mRNA levels in the amygdala, mPFC, and hippocampus and fear behavior of the dogs.

Two SNPs were identified in the STMN1 transcriptional regulatory region

We assumed that genetic variations in the STMN1 transcriptional regulatory region might affect its expression level. Hence, we sequenced a 2,387 bp segment from the STMN1 transcriptional regulatory region in English Springer Spaniels and identified two genetic variations (g. -327 A > G and g. -125 C > T) (Fig 2A and 2B). The two SNPs were further genotyped in a population of 317 dogs using the PCR-SSCP technique (Fig 2C, 2D and S1 Table). The genotyping results verified the DNA sequencing results. We found that the A and C alleles were the predominant g. -327 A > G and g. -125 C > T alleles, respectively. The chi-square test showed that the two SNPs were in Hardy—Weinberg equilibrium (P > 0.05) within the analyzed population. According to the PIC classification (PIC value < 0.25, low polymorphism; 0.25 < PIC < 0.5, moderate polymorphism; 0.5 < PIC < 1.0, high polymorphism), the PIC value of g. -327 A > G and g. -125 C > T were 0.34 and 0.32, respectively.

Fig 1. STMN1 gene expression in different canine tissues. (A) Canine Stathmin 1 (STMN1) mRNA expression profiles in 18 tissue types, determined in triplicate. The mean heart level was set to 1. Different capital letters on the bars indicate significant differences (P < 0.01). (B) STMN1 expression level in the low and high fear level groups (n = 5/group) in the amygdala, brainstem, medial prefrontal cortex (mPFC), and hippocampus, respectively. The mRNA levels were normalized to β-actin mRNA levels. *P < 0.05; **P < 0.01.
value < 0.5, intermediate polymorphism; and PIC value > 0.5, high polymorphism), all loci exhibited intermediate polymorphism at the STMN1 in the analyzed population (Table 2).

**Association between the two STMN1 SNPs and fear behavior in English Springer Spaniels**

The effects of the two SNPs on the fear level of 317 dogs are summarized in Table 3. The results suggest that the g. -125 C > T polymorphism was associated with fear level (P < 0.05). Animals with the g. -125 C > T TT genotype had an obviously greater fear level than those with the CC

---

### Table 2. Genotype distribution, allelic frequencies, and genetic diversity of the two single nucleotide polymorphisms (SNPs) in canine stathmin 1 (STMN1).

| SNP       | Sample size | Genotype frequencies | Allele frequencies | He   | Ne   | PIC  | p-value ($\chi^2$, HWE) |
|-----------|-------------|----------------------|--------------------|------|------|------|-------------------------|
| g. -327 A > G | 317         | AA (0.3028) AG (0.4732) GG (0.223) | A (0.5394) G (0.4606) | 0.4732 | 1.9019 | 0.3734 | 0.3696                  |
| g. -125 C > T | 317         | GG (0.2776) CT (0.5426) TT (0.1798) | C (0.5488) T (0.4511) | 0.5426 | 2.1862 | 0.3726 | 0.0858                  |

HWE, Hardy-Weinberg equilibrium.

---

### Table 3. Least squares mean (LSM) ± standard error for the fear level of different STMN1 SNPs in English Springer Spaniel.

| SNP       | Genotype | Sample size | Fear level* |
|-----------|----------|-------------|-------------|
| g. -327 A > G | AA       | 96          | 2.3594 ± 0.1273 |
|           | AG       | 150         | 2.6133 ± 0.1105 |
|           | GG       | 71          | 2.6901 ± 0.1612 |
| g. -125 C > T | CC       | 88          | 2.2955 ± 0.1401b |
|           | CT       | 172         | 2.5814 ± 0.1026 ab |
|           | TT       | 57          | 2.8684 ± 0.1625a |

*Values with different lowercase superscripts in the same column are significantly different (P < 0.05).
genotype ($P < 0.05$), whereas the g. -327 A $>$ G genotype had no relationship with fear ($P > 0.05$).

**Association between STMN1 haplotype combinations and fear behavior in English Springer Spaniels**

Four haplotypes were constructed from the two detected SNPs: H1 (AC), H2 (AT), H3 (GC), and H4 (GT). Consequently, there are nine combinations for the population analysis: H1H1 (ACAC), H1H2 (ACAT), H1H3 (ACGC), H1H4/H2H3 (ACGT/ATGC), H2H2 (ATAT), H2H4 (ATGT), H3H3 (GCGC), H3H4 (GCGT), and H4H4 (GTGT). The effects of the nine haplotype combinations on fear level are summarized in Table 4. The results show that fear level in dogs with the H4H4 (GTGT) haplotype combination was dramatically higher than that of dogs with any of the other haplotype combinations.

**Promoter activity of the STMN1 transcriptional regulatory region**

To determine whether the -2,304 nt to +83 nt fragment had an active promoter, we amplified this 2,387 bp fragment containing the STMN1 transcriptional regulatory region (Fig 3A). Subsequently, we generated truncated constructs (P-F1, P-F2, P-F3, P-4, P-F5, and P-F6) by progressive deletion of nucleotides from the 5' -end, cloned these fragments into the pGL3-basic luciferase vector (Fig 3B), and transiently transfected them into 293T cells. As shown in Fig 3C, the promoter activity of all other constructs, except that of P-F1 and P-F3, was dramatically higher than that of the control pGL3 vector, and P-F6 was the highest. This result indicates that the fragment from -312 nt to +83 nt determines most of the STMN1 promoter activity. Therefore, the P-F6 fragment of the STMN1 proximal transcriptional regulatory region was the active core promoter. The P-F6 fragment only had g. -125 C $>$ T, whereas the P-F5 fragment had both g. -327 A $>$ G and g. -125 C $>$ T. Of note, P-F5 promoter activity was lower than that of P-F6 and was significantly higher than that of all other constructs.

**Different transcriptional activity among the STMN1 promoter haplotypes**

To investigate the impact of the potential functional SNPs on STMN1 expression, the STMN1 promoter containing g. -327 A $>$ G and g. -125 C $>$ T was further analyzed on promoter activity. Four haplotype constructs (H1: AC, H2: AT, H3: GC, and H4: GT) were used to assess the effect...
of different promoter haplotypes on STMN1 transcriptional activity (Fig 4A and 4B). The different haplotype constructs were transiently transfected into 293T cells, with the pGL3-basic vector as the control. As shown in Fig 4C, all constructs exhibited higher levels of luciferase expression than the control (P < 0.05). The H4 haplotype showed 81% and 64% higher transcriptional activity than the haplotypes H2 and H1 (P < 0.05), but there was no significant difference between the H4 haplotype and H3 haplotype. Given that the -592 nt and +83 nt region

Fig 3. Scheme of the STMN1 gene 5′-flanking region and identification of the core promoter region. (A) 5′-flanking region of canine STMN1, as identified in the National Center for Biotechnology Information (NCBI) database. Grey box represents exon 1. Other numbers represent primer positions for cloning reporter constructs. (B) Fragments P-F1, P-F2, P-F3, P-F4, P-F5, and P-F6 were amplified by PCR to produce the reporter constructs; their positions are shown in parentheses. (C) Relative luciferase activity of a series of truncated constructs in the STMN1 5′-flanking region, as measured by dual luciferase assays in 293T cells. Results are firefly luciferase activity normalized to Renilla luciferase activity for each sample. The pGL3-basic reporter vector was used as a control. *P < 0.05; **P < 0.01.

doi:10.1371/journal.pone.0158756.g003

Fig 4. STMN1 promoter transcriptional activity and the recombinant haplotypes. (A) 5′-flanking region of canine STMN1, as identified using the NCBI database. Grey box represents exon 1. g. -327 A > G and g. -125 C > T are located in the STMN1 5′-flanking region (~−2,304 nt to +83 nt). Other numbers represent primer positions for the cloning reporter constructs. (B) The P-F5 fragment with different haplotypes (H1, H2, H3, and H4) was amplified by polymerase chain reaction to generate the reporter constructs; the various recombinant haplotypes are shown above the line. Each fragment was cloned into the pGL3 basic vector and transfected into 293T cells. (C) STMN1 promoter transcriptional activities with various haplotypes were measured by dual luciferase assays. Results are normalized firefly luciferase activity to Renilla luciferase activity for each sample. The pGL3-basic reporter vector was used as a control. Compared with the basal activity of control, the promoter activity of all haplotype constructs was higher (*P < 0.05). Compared with the H4 activity, H1 and H2 showed significant lower activity (#P < 0.05).

doi:10.1371/journal.pone.0158756.g004
is the core promoter, we speculate that g. -327 A>G and g. -125 C>T may be related in transcriptional regulation of STMN1. Overall, our results indicate that the functional g. -327 A>G and g. -125 C>T variants in the recombined haplotypes play crucial roles in STMN1 promoter.

Transcription factor prediction analysis for the STMN1 core promoter region

The bioinformatics and prediction analysis indicated that the STMN1 core promoter region was located in the -477 nt to +139 nt region. The transcription factor prediction analysis showed that canine STMN1 has a typical TATA box in its promoter region. The putative promoter contained several functional elements, including POU6F1, SRY, c-Ets-2, GATA-3, SP2, MZF1, and E2F7 transcription factor binding sites. Both g. -327 A>G and g. -125 C>T located in the transcription factor binding site of the promoter core region. However, the presence of a mutant G allele at g. -327 A>G created the polyamine-modulated factor 1 (PMF1) (-) transcription factor binding motif, whereas the presence of a mutant T allele at g. -125 C>T created NK6 homeobox 3 (Nkx6.3) (+) and FOXP1 (-) transcription factors binding motifs (Fig 5).

Finally, we conducted multiple sequence alignments to determine whether the STMN1 core promoter regions are evolutionally conserved across 6 species using publically available DNA sequences. The results revealed that the PMF1 binding site, which is present only in dog, is poorly conserved. The Nkx6.3 (+) motif is conserved in dog, horse and cow. The FOXP1 (-) binding site is conserved in dog, horse, cow and rat. We found that human and rodent species possessed very low levels of homology with dog in the three interest binding sites (Fig 6).

Discussion

STMN1 can bind to tubulin, inhibit MT assembly and promote MT catastrophes, which are related to fear behavior [27, 29, 31]. STMN1 is ubiquitously expressed in the rat brain and spinal cord, particularly within the pyriform, cingulate, and neocortex [40]. In adult rodents, STMN1 is highly expressed in the prefrontal cortex and nucleus accumbens [41], as well as the LA and related thalamo-cortical structures [31]. Therefore, we propose that STMN1 can affect
fear behavior in dogs. To verify our hypothesis, RT-qPCR analyses were conducted to explore the distribution of the \textit{STMN1} in 18 tissues of English Springer Spaniel. The highest \textit{STMN1} transcript levels in our samples were found in the amygdala, which is consistent with a previous study [31]. Moreover, we detected a significant positive correlation between \textit{STMN1} mRNA level and fear behavior, which is also in accordance with a previous report [42]. Taken together, these data suggest that \textit{STMN1} is an important determinant of fear behavior in English Springer Spaniels.

As \textit{STMN1} expression determined fear behavior, we hypothesized that mutations in the \textit{STMN1} promoter region would affect \textit{STMN1} expression differently between groups with high- and low-fear levels. The \textit{STMN1} rs182455 SNP, which is located within or near to the putative transcriptional control region, has been previously correlated with fear responses in healthy people [27]. Moreover, the \textit{STMN1} rs182455 C-allele was linked to cognitive-affective processing in healthy people [28]. In our study, we cloned and sequenced the transcriptional regulatory region of canine \textit{STMN1} to determine genetic variations. Two SNPs (g. -327 A> G and g. -125 C> T) were identified in the \textit{STMN1} promoter region, and animals with g. -125 C> T genotype had greater fear levels than those with the CC genotype by association analysis. The g. -125 C> T TT genotype was significantly associated with fear level, whereas the g. -327 A> G GG genotype was not. However, dogs with the H4H4 (H4 = GT) haplotype combination showed relatively higher fear levels. Therefore, the influences of the g. -327 A> G and g. -125 C> T SNPs, specifically the H4H4 haplotype combination, on fear level requires further elucidation. Overall, these results suggest that \textit{STMN1} gene is associated with canine fear behavior, and the mutation could be used as the molecular genetic marker to select working dogs and breeding dog with low fear behavior.

We explored the transcriptional activity of the \textit{STMN1} promoter region, and our results revealed that the P-F6 fragment had the highest promoter activity, which is in accordance with predictions of bioinformatic analysis. As the function of this fragment remains unclear, we speculate that upstream regulatory elements may interact with each other to regulate \textit{STMN1}. 

![Fig 6. Multiple sequence alignments of the \textit{STMN1} core promoter region among six species. (A) The black box presents PMF1 binding site. (B) The two black boxes present Nkx6.3 and FOXP1 binding site respectively. Nucleotides are numbered relative to the dog \textit{STMN1} gene transcription start site. Sequence consistent with dog \textit{STMN1} is labeled by asterisk, and a gap is represented by a hyphen (-). Nucleotides shown in bold and underlined font represent differences from the dog regulatory elements. doi:10.1371/journal.pone.0158756.g006](image-url)
expression. In addition, identifying other signals that indirectly regulate STMN1 promoter activity would be interesting. Haplotypes are more likely to affect traits than SNP [43, 44]. In this study, the g. -327 A > G and g. -125 C > T SNPs were located at the STMN1 core promoter region, indicating their possible important function in canine STMN1 expression. The H1 (AC), H2 (AT), H3 (GC), and H4 (GT) haplotypes were transfected into 293T cells to examine their impacts on STMN1 promoter transcriptional activity. We found that the pGL3-H4 reporter plasmid had the highest relative luciferase activity among all plasmids. The fact that different haplotypes exhibited different promoter activities, suggests that SNPs may regulate STMN1 expression and further affect the physiological function of STMN1 on fear behavior.

Interestingly, the g. -327 A > G and g. -125 C > T SNPs locate in the putative canine STMN1 promoter region. SNPs in gene coding regions can change the biological character of the encoded protein, while SNPs in non-coding regions may regulate gene expression in an allele-specific manner, and these regulatory polymorphisms stand for a relatively unexplored class of genetic variation. In fact, many reports found that promoter region polymorphisms regulate gene expression levels [27, 45]. Genetic variations in transcription factor binding sites may alter the binding affinity of transcription factors that control gene expression [46] and hence cause significant phenotypic diversity [47].

In our study, the existence of the mutant G allele at g. -327 A > G generated a PMF1 (-) transcription factor binding motif, and the existence of the mutant T allele at g. -125 C > T generated the Nkx6.3 (+) and FOXP1 (-) transcription factors binding motifs. PMF-1 doesn’t belong to Maf family, but does possess a DNA binding region and a binding site for the NF-E2 related factor-2 (Nrf-2) protein [48]. PMF-1 binds Nrf-2 to regulate transcription of the spermidine/spermine N⁺-acetyltransferase gene and the 4E-BP1 gene [49, 50]. Nkx6.3, a member of the NKKX6 subfamily, contains an Engrailed-homology domain that may mediate interactions with transcriptional co-repressors [51]. Studies have revealed that it is involved in development of the central nervous system (CNS) [52, 53]. FOXP1 and FOXP2 belong to the FOXP subfamily of transcription factors [54]. They form heterodimers to control transcription [55] and are co-expressed in the brain [56, 57], suggesting that they cooperate in common pathways of cognitive and language development. Collectively, we speculate that the g. -327 A > G and g. -125 C > T mutations in the STMN1 promoter region resulted in recruitment of different transcription factors, which subsequently altered gene expression. However, no direct evidence shows that these predicted transcription factors affect fear behavior. Further research is needed to reveal the molecular mechanisms involved.

In summary, our results suggest that the g. -327 A > G and g. -125 C > T SNPs of STMN1 and the H4H4 (H4 = GT) haplotype combination were associated with canine fear behavior. They were identified in the putative promoter region and affected the STMN1 transcription factor binding sites. Moreover, promoter regions with different haplotypes displayed different promoter activities, suggesting that these SNPs likely modulate STMN1 promoter binding activity and further affect canine fear level. Overall, our data suggest that variations in STMN1 affect the molecular signaling that regulates fear behavior. In addition, our findings demonstrate that the STMN1 genotype is related to the regulation of fear level in canines and the two SNPs might be used as molecular markers to select working dog and breeding dog.

Supporting Information

S1 Table. The results of fear behavioral test and SNPs genotyping in 317 English Springer Spaniels.

(XLSX)
Acknowledgments

We acknowledge the Nanjing Police Dog Institute of the Ministry of Public Security of the People’s Republic of China for the permission us to use the data in this study. A special thank to Xijun Bao, a veterinarian, for his help with collection samples. Finally, we are grateful to all of you who have contributed in the behavioural test and in the collection of these data. You made this study possible.

Author Contributions

Conceived and designed the experiments: XLD YXX. Performed the experiments: XLD JH. Analyzed the data: JH. Wrote the paper: XLD HYZ.

References

1. Alcaidinho J, Valentin G, Yoder N, Tai S, Mundell P, Jackson M. Assessment of Working Dog Suitability from Quantimetric Data. Nordic Conference on Human-Computer Interaction; 2014.
2. Wilsson E, Sundgren P-E. Behaviour test for eight-week old puppies—heritabilities of tested behaviour traits and its correspondence to later behaviour. Applied Animal Behaviour Science. 1998; 58(1):151–62.
3. Ruefenacht S, Gebhardt-Henrich S, Miyake T, Gaillard C. A behaviour test on German Shepherd dogs: heritability of seven different traits. Applied Animal Behaviour Science. 2002; 79(2):113–32.
4. Zovkic IB, Sweatt JD. Epigenetic mechanisms in learned fear: implications for PTSD. Neuropsychopharmacology. 2013; 38(1):77–93. doi: 10.1038/npp.2012.79 PMID: 22692566
5. Conley MJ, Fisher AD, Hemsworth PH. Effects of human contact and toys on the fear responses to humans of shelter-housed dogs. Applied Animal Behaviour Science. 2014; 156:62–9.
6. Jones RB, Waddington D. Modification of fear in domestic chicks, Gallus gallus domesticus, via regular handling and early environmental enrichment. Animal Behaviour. 1992; 43(6):1021–33.
7. Gray JA. The psychology of fear and stress. CUP Archive; 1987.
8. Walczak M, Adamkiewicz E, Walasek A, Lisowski P, Jeziorski T. Evaluation of fear-related aggression in police patrol dogs to unfamiliar humans during socialization stimulus. Journal of Veterinary Behavior: Clinical Applications and Research. 2014; 6(9):e13.
9. Radosta-Huntley L, Shofer F, Reisner I. Comparison of 42 cases of canine fear-related aggression with structured clinician initiated follow-up and 25 cases with unstructured client initiated follow-up. Applied Animal Behaviour Science. 2007; 105(4):330–41.
10. Morrow M, Ottobre J, Ottobre A, Neville P, St-Pierre N, Dreschel N, et al. Breed-dependent differences in the onset of fear-related avoidance behavior in puppies. Journal of Veterinary Behavior: Clinical Applications and Research. 2015; 10(4):286–94.
11. Svarberg K, Forkman B. Personality traits in the domestic dog (Canis familiaris). Applied animal behaviour science. 2002; 79(2):133–55.
12. Ley J, Coleman GJ, Holmes R, Hemsworth PH. Assessing fear of novel and startling stimuli in domestic dogs. Applied Animal Behaviour Science. 2007; 104(1):71–84.
13. Araujo JA, de Rivera C, Landsberg GM, Adams PE, Milgram NW. Development and validation of a novel laboratory model of sound-induced fear and anxiety in Beagle dogs. Journal of Veterinary Behavior: Clinical Applications and Research. 2013; 8(4):204–12.
14. King T, Hemsworth P, Coleman G. Fear of novel and startling stimuli in domestic dogs. Applied Animal Behaviour Science. 2003; 82(1):45–64.
15. Jones AC, Gosling SD. Temperament and personality in dogs (Canis familiaris): a review and evaluation of past research. Applied Animal Behaviour Science. 2005; 95(1):1–53.
16. Tiira K, Lohi H. Reliability and validity of a questionnaire survey in canine anxiety research. Applied Animal Behaviour Science. 2014; 155:82–92.
17. Wilsson E, Sinn DL. Are there differences between behavioral measurement methods? A comparison of the predictive validity of two ratings methods in a working dog program. Applied Animal Behaviour Science. 2012; 141(3):158–72.
18. van den Berg L, Imholz S, Versteeg SA, Leegwater PA, Zijlstra C, Bosma AA, et al. Isolation and characterization of the canine serotonin receptor 1B gene (htr1B). Gene. 2004; 326:131–9. PMID: 14729271
19. Ozon S, Maucuer A, Sobel A. The stathmin family. European Journal of Biochemistry. 1997; 248 (3):794–806. PMID: 9342231
20. Ozon S, Byk T, Sobel A. SCLIP: A Novel SCG10-Like Protein of the Stathmin Family Expressed in the Nervous System. Journal of neurochemistry. 1998; 70(6):2386–96. PMID: 9603203
21. Stein R, Mori N, Matthews K, Lo L-C, Anderson DJ. The NGF-inducible SCG10 mRNA encodes a novel membrane-bound protein present in growth cones and abundant in developing neurons. Neuron. 1988; 1(6):463–76. PMID: 3272176
22. Maucuer A, Moreau J, Mechali M, Sobel A. Stathmin gene family: phylogenetic conservation and developmental regulation in Xenopus. Journal of Biological Chemistry. 1993; 268(22):16420–9. PMID: 8344928
23. Belmont LD, Mitchison TJ. Identification of a protein that interacts with tubulin dimers and increases the catastrophe rate of microtubules. Cell. 1996; 84(4):623–31. PMID: 8598048
24. Bräuer A, Savaskan N, Plaschke M, Ninnemann O, Nitsch R. Perforant path lesion induces up-regulation of stathmin messenger RNA, but not SCG10 messenger RNA, in the adult rat hippocampus. Neuroscience. 2001; 102(3):515–26. PMID: 11226690
25. Shumyatsky GP, Tsvetkov E, Malleret G, Vronskaya S, Hatton M, Hampton L, et al. Identification of a signaling network in lateral nucleus of amygdala important for inhibiting memory specifically related to learned fear. Cell. 2002; 111(6):905–18. PMID: 12526815
26. Brocke B, Lesch KP, Armbruster D, Moser DA, Müller A, Strobel A, et al. Stathmin, a gene regulating neural plasticity, affects fear and anxiety processing in humans. American Journal of Medical Genetics Part B: Neuropsychiatric Genetics. 2010; 153(1):243–51.
27. Ehlis AC, Bauernschmitt K, Dresler T, Hahn T, Herrmann MJ, Röser C, et al. Influence of a genetic variant of the neuronal growth associated protein Stathmin 1 on cognitive and affective control processes: An event-related potential study. American Journal of Medical Genetics Part B: Neuropsychiatric Genetics. 2011; 156(3):291–302.
28. Martel G, Nishi A, Shumyatsky GP. Stathmin reveals dissociable roles of the basolateral amygdala in parental and social behaviors. Proceedings of the National Academy of Sciences. 2008; 105 (38):14620–5.
29. Martel G, Hevi C, Wong A, Zushida K, Uchida S, Shumyatsky GP. Murine GRPR and stathmin control in opposite directions both cued fear extinction and neural activities of the amygdala and prefrontal cortex. PLOS One. 2012; 7(2):e30942. doi: 10.1371/journal.pone.0030942 PMID: 22312434
30. Shumyatsky GP, Malleret G, Shin R-M, Takizawa S, Tully K, Tsvetkov E, et al. Stathmin, a gene enriched in the amygdala, controls both learned and innate fear. Cell. 2005; 123(4):697–709. PMID: 16286011
31. Hydbring-Sandberg E, von Walter LW, H02glund K, Svartberg K, Swenson L, Forkman B. Physiological reactions to fear provocation in dogs. Journal of Endocrinology. 2004; 180(3):439–48. PMID: 15012598
32. Goddard M, Beilharz R. A multivariate analysis of the genetics of fearfulness in potential guide dogs. Behavior Genetics. 1985; 15(1):69–89. PMID: 3985912
33. Hu J, Yu P, Ding X, Xu M, Guo B, Xu Y. Genetic polymorphisms of the AMPD1 gene and their correlations with IMP contents in Fast Partridge and Lingshan chickens. Gene. 2015; 574(2):204–10. doi: 10.1016/j.gene.2015.08.008 PMID: 26275943
34. Pan Q, Ju Z, Huang J, Zhang Y, Qi C, Gao Q, et al. PLCz functional haplotypes modifying transcriptional activity are associated with semen quality traits in Chinese Holstein bulls. PLOS one. 2013; 8(3):e58795. doi: 10.1371/journal.pone.0058795 PMID: 23554927
35. Fu Y, Sun W, Xu C, Gu S, Li Y, Liu Z, et al. Genetic variants in KDR transcriptional regulatory region affect promoter activity and intramuscular fat deposition in Erhualian pigs. Animal genetics. 2014; 45 (3):373–80. doi: 10.1111/age.12148 PMID: 24673468
36. Hoshino S, Kimura A, Fukuda Y, Dohi K, Sasazuki T. Polymerase chain reaction—single-strand conformation polymorphism analysis of polymorphism in DPA1 and DPB1 genes: a simple, economical, and rapid method for histocompatibility testing. Human immunology. 1992; 33(2):98–107. PMID: 13487443
37. Liu K, Muse SV. PowerMarker: an integrated analysis environment for genetic marker analysis. Bioinformatics. 2005; 21(9):2128–9. PMID: 15705655
38. Yu S, Chu W, Zhang L, Han H, Zhao R, Wu W, et al. Identification of Laying-Related SNP Markers in Geese Using RAD Sequencing. PLOS One. 2015; 10(7):e0131572. doi: 10.1371/journal.pone.0131572 PMID: 26181055
40. Peschanski M, Hirsch E, Dusart I, Doye V, Marty S, Manceau V, et al. Stathmin: cellular localization of a major phosphoprotein in the adult rat and human CNS. Journal of Comparative Neurology. 1993; 337(4):655–68. PMID: 8288776

41. Hayashi K, Pan Y, Shu H, Ohshima T, Kansy JW, White CL, et al. Phosphorylation of the tubulin-binding protein, stathmin, by Cdk5 and MAP kinases in the brain. Journal of neurochemistry. 2006; 99(1):237–50. PMID: 16925597

42. Zhang L, Feng D, Tao H, De X, Chang Q, Hu Q. Increased stathmin expression strengthens fear conditioning in epileptic rats. Biomedical reports. 2015; 3(1):28–32. PMID: 25469242

43. Capparelli R, Parlato M, Amoroso M, Roperto S, Marabelli R, Roperto F, et al. Mannose-binding lectin haplotypes influence Brucella abortus infection in the water buffalo (Bubalus bubalis). Immunogenetics. 2008; 60(3–4):157–65. doi:10.1007/s00251-008-0284-4 PMID: 18330558

44. Fallin D, Cohen A, Essioux L, Chumakov I, Blumenfeld M, Cohen D, et al. Genetic analysis of case/control data using estimated haplotype frequencies: application to APOE locus variation and Alzheimer's disease. Genome research. 2001; 11(1):143–51. PMID: 11156623

45. Madsen HO, Satz ML, Hogh B, Svejgaard A, Garred P. Different molecular events result in low protein levels of mannan-binding lectin in populations from southeast Africa and South America. The Journal of Immunology. 1998; 161(6):3169–75. PMID: 9743385

46. Chorley BN, Wang X, Campbell MR, Pittman GS, Noureddine MA, Bell DA. Discovery and verification of functional single nucleotide polymorphisms in regulatory genomic regions: current and developing technologies. Mutation Research/Reviews in Mutation Research. 2008; 659(1):147–57.

47. Wang X, Tomso DJ, Liu X, Bell DA. Single nucleotide polymorphism in transcriptional regulatory regions and expression of environmentally responsive genes. Toxicology and applied pharmacology. 2005; 207(2):84–90. PMID: 16002116

48. Wang Y, Devereux W, Stewart T, Casero R Jr. Characterization of the interaction between the transcription factors human polyamine modulated factor (PMF-1) and NF-E2-related factor 2 (Nrf-2) in the transcriptional regulation of the spermidine/spermine N1-acetyltransferase (SSAT) gene. Biochem J. 2001; 355:45–9. PMID: 11256947

49. Stephenson A, Seidel E. Analysis of the interactions of Nrf-2, PMF-1, and CSN-7 with the 5'-flanking sequence of the mouse 4E-BP1 gene. Life sciences. 2006; 79(13):1221–7. PMID: 16647090

50. Wang Y, Devereux W, Stewart TM, Casero RA. Cloning and characterization of human polyamine-modulated factor-1, a transcriptional cofactor that regulates the transcription of the spermidine/spermine N 1-acetyltransferase gene. Journal of Biological Chemistry. 1999; 274(31):22095–101. PMID: 10419538

51. Muhr J, Andersson E, Persson M, Jessell TM, Ericson J. Groucho-mediated transcriptional repression establishes progenitor cell pattern and neuronal fate in the ventral neural tube. Cell. 2001; 104(6):861–73. PMID: 11290324

52. Alanentalo T, Chatonnet F, Karlen M, Sulniute R, Ericson J, Andersson E, et al. Cloning and analysis of Nkx6.3 during CNS and gastrointestinal development. Gene expression patterns. 2006; 6(2):162–70. PMID: 16326147

53. Hafler BP, Choi MY, Shvidasani RA, Rowitch DH. Expression and function of Nkx6.3 in vertebrate hindbrain. Brain research. 2008; 1222:42–50.

54. Banham AH, Beasley N, Campo E, Fernandez PL, Fidler C, Gatter K, et al. The FOXP1 winged helix transcription factor is a novel candidate tumor suppressor gene on chromosome 3p. Cancer research. 2001; 61(24):8820–9. PMID: 11751404

55. Li S, Weidenfeld J, Morrissey EE. Transcriptional and DNA binding activity of the Foxp1/2/4 family is modulated by heterotypic and homotypic protein interactions. Molecular and cellular biology. 2004; 24(2):809–22. PMID: 14701752

56. Ferland RJ, Cherry TJ, Prevare PO, Morrissey EE, Walsh CA. Characterization of Foxp2 and Foxp1 mRNAs and protein in the developing and mature brain. Journal of comparative Neurology. 2003; 460(2):266–79. PMID: 12687690

57. Teramitsu I, Kudo LC, London SE, Geschwind DH, White SA. Parallel Foxp1 and Foxp2 expression in songbird and human brain predicts functional interaction. The Journal of Neuroscience. 2004; 24(13):3152–63. PMID: 15056695