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A novel BDNF polymorphism affects plasma protein levels in interaction with early adversity in rhesus macaques

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Summary

Early stressful events can increase vulnerability for psychopathology, although knowledge on the effectors is still limited. In this report we describe the characterization of a single nucleotide polymorphism (SNP) in rhesus macaques, which results in a Val to Met transition in the pro-BDNF domain, similar to a well described variant in the human gene. Further, we tested the hypothesis that peripheral levels of BDNF, which is involved in the response to stress and in the pathophysiology of anxiety and depression, might be differentially affected in a non-human primate model of early adverse rearing in a genotype-dependent manner. Males and females rhesus macaques reared either with their mothers (MR), in peer-only groups (PR), or in a "surrogate/peer-reared" (SPR) condition with limited peer interactions, were used as experimental subjects. BDNF levels were determined at baseline on postnatal days (PND) 14, 30 and 60 by means of specific ELISA procedure. Data indicate that BDNF levels were increased as a result of peer-rearing and that this increase was moderated by the presence of the SNP. Overall these data indicate that a SNP, which results in a Val to Met transition in the pro-BDNF domain, is present in rhesus macaques and is able to affect BDNF peripheral levels, thus making this primate model a fundamental tool to study gene by environment interactions involving the BDNF gene.

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Conflict of interest

All authors declare they have no conflict of interest.

Keywords

Brain-derived neurotrophic factor (BDNF); polymorphism; stress; depression; non-human primates

1. Introduction

Early adverse experiences in humans are associated with an increased risk for developing psychiatric disorders such as anxiety and major depression (Kaufman et al., 2000; McEwen, 2000; Heim and Nemeroff, 2001), although little is known of the neurobiological mediators. Brain-Derived Neurotrophic Factor (BDNF), a neurotrophin which is affected by stress and plays a fundamental role in brain functions and neuroprotection, is a good candidate for mediating the effects of adverse life events into changes in brain functions (Smith et al., 1995; Thoenen, 1995; Duman et al., 1997; Martinowich et al., 2007). Neurotrophins are also produced by cells outside the nervous system, thus being in a position to integrate neural, immune and endocrine responses to stress (Aloe et al., 1986; Nisoli et al., 1996; Nockher and Renz, 2005).

Both genetic and experiential factors can contribute to the development of psychopathology (Yehuda et al., 1997; Heim and Nemeroff, 1999). In one of the first studies involving gene-environment interactions, Caspi and coworkers (Caspi et al., 2003) showed that a functional polymorphism in the promoter region of the serotonin transporter gene (5-HTT-LPR) moderates the influence of stressful life events on vulnerability to depression, i.e. provided the first evidence for gene × environment (G × E) interaction in psychiatric disorders. Individuals with one or two copies of the 5-HTT 'short' allele exhibited more depressive symptoms, diagnosable depression and suicidality following stressful life events than individuals with two copies of the 'long' allele (Caspi et al., 2003). A considerable number of replication studies have been published since that initial paper; although one meta-analysis did not provide unequivocal evidence of 5-HTT × E (Risch et al., 2009), critical appraisal of all pertinent data (Uher and McGuffin, 2008; Huezo-Diaz et al., 2009; Caspi et al. 2010) suggests that 5-HTT-LPR indeed has a role in translating environmental adversities to human behavior, and non-human primate studies have been shown to have predictive validity for examining GxE interactions (Barr et al., 2004; Caspi et al., 2010). Although 5-HTT-LPR genotype may explain a portion of GxE effects, other genes, most likely, have to play a role as well.

An increasing number of studies use serum BDNF levels as a potential indicator for central nervous system alterations as changes in peripheral levels of this neurotrophin have been associated with mood disorders, also in interaction with early trauma (Aloe et al., 1994; Hadjiconstantinou et al., 2001; Karege et al., 2002; Karege et al., 2005; Kaufman et al., 2006; Castren et al., 2007; Kauer-Sant'Anna et al., 2007; Mitoma et al., 2008). In addition, recent data on a rat model of electroconvulsive treatment has provided substantial evidence that it can be justified to measure serum BDNF levels as indices of central activity, although changes in neurotrophin levels might show a different time course in the brain and periphery (Sartorius et al., 2009).

Haploinsufficiency of BDNF goes along with decreased peripheral BDNF levels as well as childhood-onset obesity (Han et al., 2008). Although this genetic variant is rare, as are several other coding region variants (Licinio et al., 2009), there is also a frequent non-synonymous single-nucleotide polymorphism (SNP), which results in an aminoacid substitution in the pro-BDNF domain (rs6265, Val66Met). Met allele carriers have attenuated intracellular trafficking and secretion of BDNF and show comparatively lower

hippocampal gray matter and poorer cognitive performance (Egan et al., 2003; Chen et al., 2004). Not surprisingly, this SNP has been tested for association with a wide range of psychiatric disorders. Rs6265 has been associated with substance abuse, eating disorders and schizophrenia (Verhagen et al., 2010). G × E interactions might add a further level of complexity, as it was shown that the Met allele interacts with severe life events thereby causing psychiatric symptoms (Kaufman et al., 2000; Savitz et al., 2007; Elzinga et al., 2010), also in interaction with 5-HTT-LPR (Kaufman et al., 2006; Wichers et al., 2008).

In line with the notion that the Met allele is implicated in the etiopathology of depression, it was shown that Met allele carriers, irrespective of diagnosis, have a decreased hippocampal volume (Frodl et al., 2007), which might also provide a neuroanatomical basis for G × E effects as Met allele carriers have a smaller hippocampal volume only in the presence of severe life events (Gatt et al., 2009). A similar mechanism has already been proposed for post-traumatic stress disorder (Gross and Hen, 2004).

Taken together, there is thus ample evidence that rs6265 (a) predicts peripheral BDNF levels and (b) interacts with life stress to increase the risk for depression, making this SNP a prime biomarker for susceptibility to psychopathology in humans, including depression. The precise interplay between rs6265 and life stress however cannot readily be tested in humans and strategies, such as variant BDNF mouse models (BDNF Met/Met) that reproduce phenotypic hallmarks characterizing humans with the variant allele (Chen et al., 2006), although of great advantage, bear the disadvantage of suboptimal behavioral repertoires which do not mirror well human psychopathology.

We have previously shown in a non-human primate model that maternal deprivation with some form of social contact, such as access to peers, leads to important emotional and social disturbances and behavioral abnormalities, such as motor stereotypies (Suomi, 1991; Champoux et al., 2002; Barr et al., 2003). Peer-reared macaques develop inadequate social skills, are highly reactive and aggressive and, as adults, show increased voluntary alcohol consumption, and typically are low-ranking in mixed social groups (Suomi, 1991; Barr et al., 2003). When peer interactions are limited in time, the effects are even more pronounced. We have recently shown that peripheral levels of BDNF are able to track these behavioral changes (Cirulli et al., 2009b). In particular, we have shown that a selective increase in BDNF peripheral levels occurs in response to early adversity caused by peer-rearing.

In this paper we report for the first time that a polymorphism of the BDNF gene (Val66Met) interacts with early social rearing adversity and results in lower peripheral BDNF levels in rhesus macaques.

2. Methods

2.1. Animals and rearing procedures

Subjects of these studies were 19 males and 18 females rhesus monkey infants (*Macaca mulatta*) born between 2003 and 2005 in the Laboratory of Comparative Ethology, NICHD breeding facility at the NIH Animal Center near Poolesville (MD, USA). Thirteen subjects were “mother-reared” (MR), raised in social groups either by their biological mothers or by an unrelated multiparous foster mother. Thirteen others infants were reared without adults, but with constant access to age-mate peers in a “peer-only reared” (PR) condition, while an additional 11 infants were reared with inanimate surrogates and limited peer interactions in a “surrogate/peer-reared” (SPR) condition. Rearing conditions were randomly assigned at the time of birth balancing the number of males and females in each rearing condition.

MR infants remained with their mothers in a stable social group of 8-10 adults and peers. Infants assigned to the PR and SPR conditions were separated from their mothers at birth, and were subsequently hand-reared in a neonatal nursery. During the first 37 days of their life, PR and SPR infants were treated in an identical manner. For the first 14 days, they were kept in an incubator and hand-fed. Each cage contained a blanket and a terry cloth-covered rocking "surrogate" covered by a heating pad. The infant could see and hear, but did not experience any physical contact with the other infants. From day 15 until day 38, infants were moved with their surrogate in individual nursery cages. At 38 days of age all nursery-reared infants were placed into their final condition (PR or SPR). PR infants were placed in permanent social groups of 3-4 age-mates, similarly-reared peers, with whom they had continuous contact. SPR monkeys differed from PR monkeys only in the amount of time that they were allowed to interact with their age-mates each day. SPR infants continued to be housed individually with their surrogates, being provided with only limited peer contact (2 hours/day, 5 days/week). This procedure allowed the infants to socialize in the absence of a mother, without allowing them to become overly attached to one another (cf. Shannon et al., 1998) for additional details regarding these respective rearing procedures).

Protocols for the use of experimental animals were approved by the Institutional Animal Care and Use Committee of the NICHD. All animal experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. DNA extraction and genotyping

In order to test whether the human polymorphisms rs6265 is also present in rhesus monkeys, DNA from the above subjects was amplified by PCR using the forward primer CAAACATCCGAGGACAAGGT and the reverse primer AGAAGAGGAGGCTCCAAAGG, thereby generating an amplicon of 250 bp covering rs6265 in humans, as tested in four previously genotyped human control subjects, and rhesus monkeys based on the UCSC database (Jan. 2006 [MGSC Merged 1.0/rheMac2] assembly). PCR was carried out with 50 mM KCl, 0.025% Tween20, 1.5mM $MgCl_2$. 200 μM dNTPs, 0.2 μM primers dissolved in 10 mM Tris-HCl (pH=8.3). PCR conditions were 2 min at 96°C, and 30 cycles of 20 sec at 96°C, 20 sec at 58°C, and 30 sec at 72°C. Amplicons were treated with exonuclease and phosphatase (ExoSapIT, Amersham) and sequenced thereafter using the QuickStart Sequencing-Kit (Beckman-Coulter, Krefeld, Germany) on a Beckman CEQ8000 sequencer (Beckman-Coulter, Krefeld, Germany).

2.3. BDNF measurement

On days 14 and 30 after standardized behavioral testing (Schneider et al., 1991), animals were anesthetized with ketamine hydrochloride (intramuscularly, 10 mg/kg), and blood was drawn from the femoral vein in EDTA-coated tubes. After centrifugation, a 500 μl aliquot of plasma was placed into a plastic vial and stored at −70°C until assay. On PND 60 another baseline measure was taken. The same sampling and processing procedures were used for all groups.

Plasma was assayed for levels of BDNF by highly sensitive immunoenzymatic assays, following the procedure suggested by the manufacturer (Emax^{tm} ImmunoAssay System number G6891, Promega, Madison, WI, USA; Aloe et al., 1994). A monoclonal anti-mouse-BDNF antibody and a polyclonal anti-human-BDNF antibody were used. BDNF concentration was determined from the regression line for the BDNF standard curve (ranging from 7.8 to 500 pg/ml-purified mouse BDNF) incubated under similar conditions in

each assay. The sensitivity of the assay is about 15 pg/ml of BDNF and the cross-reactivity with other related neurotrophic factors (NGF, NT-3, and NT-4) is considered nil.

Our sampling methodology (which was the same for all experimental groups) can be considered not to be biased by platelet reactivity and reflects circulating levels of BDNF (see also Cirulli et al., 2009b) for more details).

2.4. Statistical analysis

StatView 5.0.1 (SAS Institute, Inc., Cary, NC) was used for all statistical analyses. Mixed design, repeated measures ANOVAs were performed on data to assess the effect of genotype, age and rearing condition, but not gender, and their interactions. Since only three cases analyzed were homozygous for the BDNF gene variant described, these were considered together with heterozygous cases. Tukey tests were used for *post-hoc* comparisons.

3. Results

3.1. Characterization of a novel SNP in the BDNF gene in rhesus macaques

Although SNPs are not generally conserved between rhesus and humans, there are instances in which functionally similar variants occur in the two species (see for example Miller et al., 2004; Barr et al., 2008). To determine whether there were any SNPs in macaques that were functionally similar to the human Val66Met SNP, we sequenced the 250 bp genomic region flanking the orthologous site in rhesus monkeys. Sequencing revealed that all cases were homozygous for the G allele at position 196, arguing against the presence of a polymorphic variant at this site. However, we detected a polymorphic site at p.136 (G>A; Fig. 1A and B), which results in a Val to Met transition in the BDNF pro-domain at codon 46.

3.2. Effects of age, genotype and rearing condition on plasma BDNF levels of rhesus monkey infants

Regardless of genotype and rearing condition, age affected levels of BDNF (main effect on age, F (2,46) = 5.236, p = 0.0089). In particular *post-hoc* comparisons showed that subjects in the PND 14 group were characterized by higher levels of BDNF, this neurotrophin decreasing over time from PND14 to PND 60 (*post-hoc* comparisons: PND 14 *vs* PND 30 and PND 60, $p < 0.01$) (Figure 2).

3.3. Effects of genotype and rearing condition in rhesus monkey infants on plasma BDNF levels

Genotype and rearing conditions *per se* also affected BDNF levels (main effects of genotype: F (1,23) = 4.472, p = 0.0455 and of rearing condition: F (2,23) = 6.071, p = 0.0076). In particular: the GG group showed higher BDNF levels than the GA-AA, while PR subjects were characterized by higher BDNF levels when compared both to the MR and the SPR groups (*post-hoc* comparisons: $p < 0.01$).

A significant interaction between genotype and rearing condition was also found (F (2,23) = 5.299, p = 0.0128). In particular PR_{GG} showed higher BDNF levels when compared to MR_{GG}, SPR_{GG} and $PR_{AA\text{-}AG}$ (*post-hoc* comparisons: $p < 0.01$; see Figure 3). Genotype and age did not significantly interact between each other to affect BDNF levels (F (2,46) = 0.852, p = 0.4333).

4. Discussion

This study describes for the first time a rhesus macaque SNP that produces a Val to Met transition in the pro-BDNF domain. This polymorphism affects peripheral BDNF levels in a G × E manner, with Met allele carriers which were peer-reared displaying significantly lower peripheral levels of this neurotrophin as compared to Val allele carriers raised the same way.

Animal models have provided supportive evidence that neurotrophins are sensitive to manipulations of the mother-infant relationship and, more in general, of the rearing environment (Cirulli et al., 1998; Cirulli, 2003a; Branchi et al., 2006; Cirulli et al., 2007). In a previous study we have shown that peer-rearing leads to increased peripheral BDNF levels in rhesus macaques, especially in females (Cirulli et al. 2009). In the present study, a further rearing condition was used, the surrogate/peer-reared condition or SPR. Subjects raised under this condition did not show an elevation in BDNF levels as the PR animals did. We hypothesize that the lack of a BDNF increase in the SPR group, compared to the peer-reared, could be a marker of unsuccessful coping, as we know that they show suppressed neuroendocrine activity (Shannon et al., 1998). Indeed, as compared to peer-rearing, the SPR condition involves reduced peer interactions and can thus be considered to be more adverse for the infants, who nonetheless grow up and establish social relationships, although often ranking at the bottom of the social hierarchy at adulthood (Bastian et al., 2003). It is possible to hypothesize that, while moderate adversity will activate a coping response (higher BDNF levels; see Cirulli et al., 2009b), a more severe situation might be characterized by an inability to mount a stress response, or even result in a suppression of BDNF gene expression, testifying an inability to face the stress of being reared in the SPR group. Since PR subjects are characterized by a greater amount of social interactions between peers than SPR, this result indirectly suggests that social relationships might represent an important buffer for highly stressful situations early during postnatal life.

Even more interesting, however, is the finding that the presence of the Met allele prevented any elevation in BDNF levels in the PR group. These data appear to be in line with previous reports indicating attenuated intracellular trafficking and secretion of BDNF in human Met allele carriers and further indicate that the effects of the gene variant might depend upon expression levels, which may be altered following stress. These data are in line with results from a variant BDNF mouse model in which the presence of the Met allele does not affect basal BDNF secretion but results in a 30% deficit in activity-dependent release of BDNF-Met from neurons (Chen et al., 2006).

Since an increase in BDNF levels is an index of a coping response to stress, we believe that the presence of the Met allele might thwart such a response, thus endangering plasticity processes (see Cirulli and Alleva, 2009 for a review). Integrating neurobiological variables (such as changes in BDNF levels) with behavioural data in subjects carrying the SNP, and exposed to different contexts, will clarify the functional significance of the BDNF polymorphism here described. Indeed the ultimate effect of a gene variant will depend upon the environmental constraints characterizing the individual. In a broader and comparative perspective, however, it is worth mentioning the positive value of genetic variation which has allowed humans and some non-human primates, such as rhesus macaques, to successfully colonize different ecological niches (Suomi, 2006).

Integrating the function of early life stress might account for inconsistencies found in the literature when investigating candidate genes or performing genome-wide association studies for affective disorders as studies examining the role of the BDNF Val66Met polymorphism on major depression are characterized by mixed results. Differences between

studies may be due to unmeasured environmental variables, including exposure to stressful stimuli (Krishnan and Taylor, 2009; Elzinga et al., 2010). Although genetic differences contribute to the vulnerability and progression of stress-related neuropsychiatric disorders, environmental factors are also important and there is evidence in humans showing that vulnerability to depression and anxiety disorders are markedly increased by early trauma (Heim and Nemeroff, 2001). It has been recently confirmed in a large epidemiological study that human Val66Met is indeed relevant for an early life GXE interaction (Elzinga et al., 2010). These authors investigated, in subjects with lifetime major depressive disorder, the impact of childhood abuse and recent life events on serum BDNF levels and examined whether BDNF Val66Met polymorphism might moderate the impact of such events. Results indicate that Met carriers are particularly sensitive to early stressful events (Elzinga et al., 2010). Data from the present study confirm in a non-human primate model these findings and suggest that a functional effect of BDNF gene variants may become manifest early on only in interaction with moderately adverse events.

Increased levels of BDNF indeed characterize acute responses to stressful events - including maternal separation - early during postnatal life, while decreased expression is more reliably found at adulthood and following chronic stress exposure (Roceri et al., 2004; Savitz et al., 2007; Cirulli et al., 2010). These data are in line with the notion that in humans, BDNF concentrations in the blood can be considered a biomarker of depression, with reduced levels of this neurotrophin characterizing depressed patients, also in interaction with early trauma (Aloe et al., 1994; Hadjiconstantinou et al., 2001; Karege et al., 2002; Karege et al., 2005; Kaufman et al., 2006; Castren et al., 2007; Kauer-Sant'Anna et al., 2007; Grassi-Oliveira et al., 2008; Mitoma et al., 2008). While animal models have provided evidence that neurotrophins are sensitive to manipulations of the mother-infant relationship and, more in general, of the rearing environment (Cirulli et al., 1998; Cirulli, 2003a; Branchi et al., 2006; Cirulli et al., 2007), early adversity, as represented by maternal separation, has been shown to increase, rather than to decrease levels of BDNF in the hippocampus of rodents as well as peripheral BDNF levels in rhesus macaques (Roceri et al., 2004). Results obtained in animal models thus suggests that changes in neurotrophin levels following early stressful events can not directly be related with neurotrophin levels in adulthood (Roceri et al., 2004).

Changes in BDNF levels following stress indicate “allostatic” processes activated to coordinate brain and body responses to specific external challenges (Chaldakov et al., 2004; McEwen, 2007; Cirulli and Alleva, 2009). In addition to acting on processes regulating neuronal growth and connectivity, neurotrophins could counteract the negative impact of stress hormones on selected brain regions, such as the hippocampus, as well as on other body organs (Thoenen, 1995). In particular, peripheral BDNF levels could mediate the response to stress through activation of peripheral tissues (Chaldakov et al., 2004; Cirulli and Alleva, 2009). However, an effect of peripheral BDNF on the central nervous system cannot be excluded since peripheral and central BDNF levels are closely related (Karege et al., 2002).

A mechanistic link between lifelong changes in behavioural traits and the establishment of permanent modifications of key genes following adverse events during the perinatal period is still lacking. Identifying a clear causal relationship between these events will require an in-depth understanding of the players and mechanisms involved, including changes taking place in chromatin and in transcriptional networks (Dulac, 2010). Among the mechanisms that have been proposed to mediate stable behavioural differences we can enlist the effect of modifications in the mother-infant relationship causing selective changes in the expression of neurotrophin genes affecting brain development in rodents and non-human primates during appropriate developmental windows (Cirulli et al., 2009a). Data presented in this

paper add to these findings suggesting that such changes might depend upon the genetic background of the individual.

One of the limitations of this study is that we could not discriminate whether the G × E effect is different in females, compared to males, due to the small sample size. Indeed, it has been previously shown that gender may play an important role in GxE interactions, as rs6265 has been selectively associated with depression in males (Verhagen et al., 2010). We are currently increasing our sample size to address this point. Indeed, based upon previous studies it is possible to hypothesize that the effect of a Val to Met transition might be more pronounced in females (Cirulli and Alleva, 2009).

The BDNF polymorphism described in this report makes this primate model a fundamental tool to study gene by environment interactions involving the BDNF gene. These studies bear important implications in order to unravel the role of this functional BDNF variation in mental health and for the characterization of the early determinants of psychopathology.

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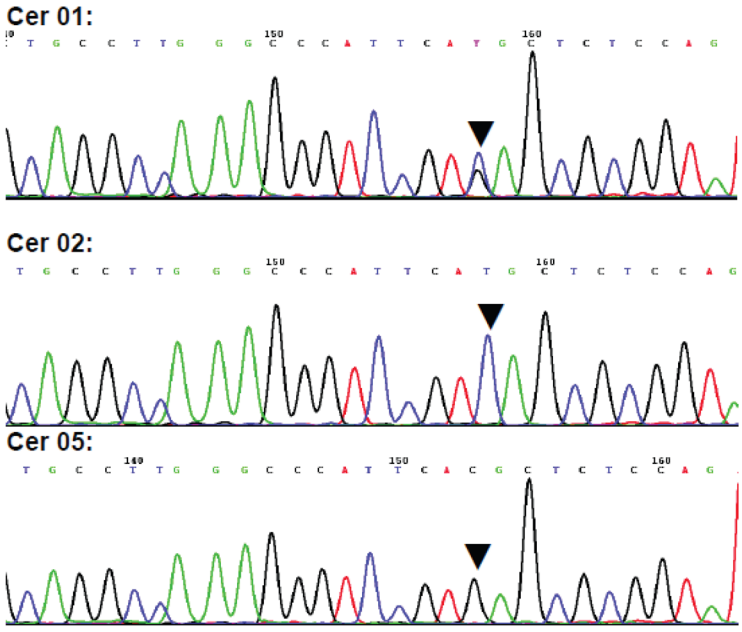

B)

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acgtccagggtgatgctcagtagtcaagtgcctttggagcctcctct      Macaca mulatta
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Figure 1.

A) Spherograms of rhesus DNA heterozygous at p.196 (Cer01), homozygous for the A allele (Cer02) and homozygous for the G allele (Cer05). B) Sequence alignment of human and rhesus DNA at the BDNF locus. Primer sequences are highlighted in yellow, and the polymorphic variants at p.136 and p.196 (G>A transitions) are printed in bold, boxed, and highlighted in green. Both variants result in a Val > Met transition at position 46 or 66, respectively, of the resulting transcript.

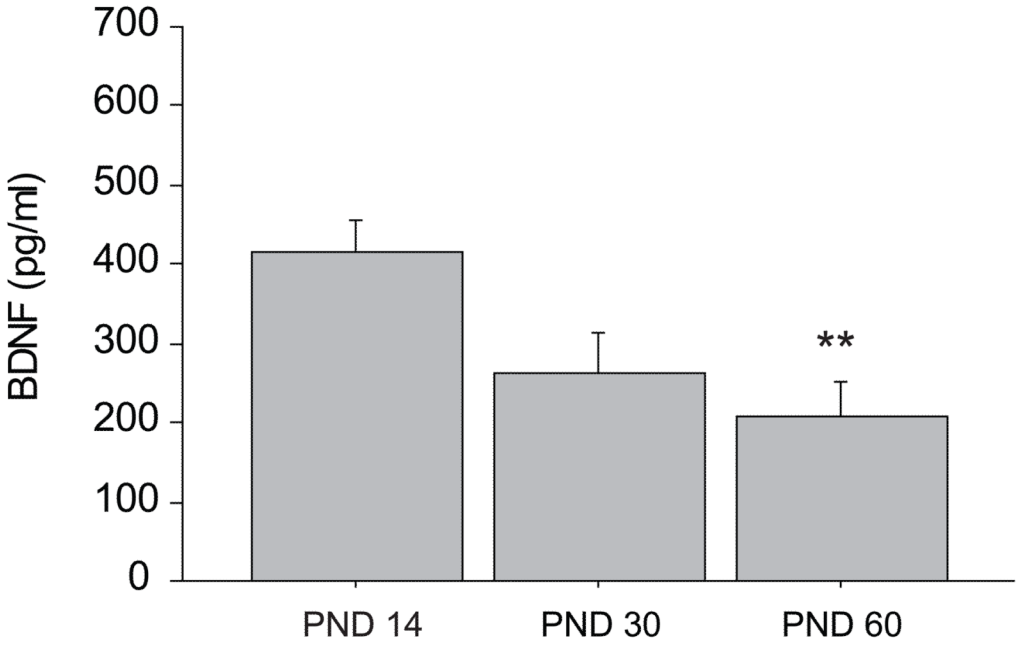

Figure 2.

Main effect of age on BDNF plasma levels in rhesus macaques. BDNF levels decreased significantly with age being higher on PND 14 compared to both PND 30 and PND 60 (** $p < 0.01$). In the graph each represented group of age is averaged over the rearing conditions and the genotype groups. Data are expressed as means (+ SEM). N = 29 subjects in each final group

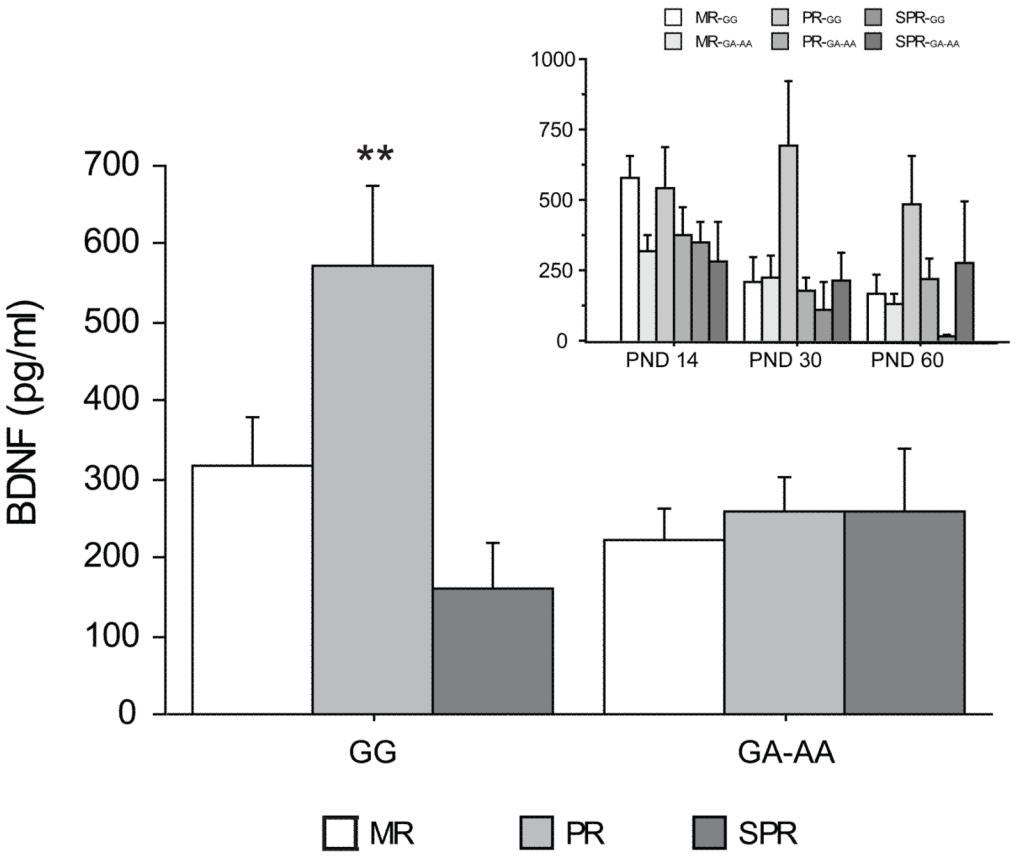

Figure 3.

Genotype interacts with rearing condition to affect BDNF protein levels in the peripheral circulation. In the PR group significantly higher BDNF levels were found in subjects with the GG genotype. This difference was abated in the AA-AG group (Tukey: PR_{GG} vs MR_{GG}, SPR_{GG} and $PR_{AA\text{-}AG}$ $p < 0.01$; ** $p < 0.01$). In the inset data are split by age. Data are expressed as means (+ SEM) of BDNF values. N = 18 (MR_{GG}); 12 (PR_{GG}); 9 (SPR_{GG}); 21 ($MR_{AA\text{-}AG}$) 18 ($PR_{AA\text{-}AG}$) 9 ($SPR_{AA\text{-}AG}$) values in each final group.