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Levels of neurotrophic factors in the hippocampus and amygdala correlate with anxiety- and fear-related behaviour in C57BL6 mice

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Summary The present study tested whether individual differences in anxiety- and fear-related behaviour are associated with between-subjects variation in postmortem brain levels of selected neurotrophic factors. Naïve C57BL6/J mice of both sexes were subjected either to an elevated plus maze test or to a Pavlovian fear conditioning paradigm. Two days after behavioural assays, the mice were sacrificed for postmortem quantification of the protein levels of brain derived neurotrophic factors (BDNF), nerve growth factor (NGF) and neurotrophin-3 (NT-3) in the hippocampus and amygdala. Significant correlations between behavioural measures and postmortem regional neurotrophic factor contents were revealed. The magnitude of anxiety-like behaviour in the elevated plus maze was positively related to dorsal hippocampal BDNF levels, but negatively related to NGF levels in dorsal hippocampus and in the amygdala. On the other hand, the expression of conditioned fear is positively related to amygdala BDNF and NGF levels, and to dorsal hippocampal NGF levels. Our results add to existing reports in human as well as in animals of correlation between anxiety trait and gross measures of hippocampal volume or activation levels. Moreover, a distinction between spontaneous and learned (or conditioned) anxiety/fear would be relevant to the identification of neurotrophin signalling mechanisms in the hippocampus and amygdala implicated in anxiety and related psychopathology.

Keywords: Brain derived neurotrophic factor, correlation, elevated plus maze, fear conditioning, nerve growth factor, neurotrophin-3, postmortem

Introduction

Neurotrophic factors were initially implicated in neuronal plasticity and development, but their functional roles in domains previously reserved for neurotransmitters have now been widely recognized (Poo, 2001). Dysfunction in neurotrophin-mediated signalling mechanisms has been implicated in the etiology and drug treatment of a number of psychiatric disorders including psychosis, depression, mania, ADHD, eating disorders and obsessive compulsive disorder (for a review, see Russo-Neustadt, 2003). There is yet no clear indication, however, that neurotrophin functions may be related to anxiety disorders although anxious traits are common amongst psychotic patients, depressives and sufferers of post-traumatic stress disorder (Weissman et al., 1993; Cosoff and Hafner, 1998; Rapaport, 2001), although neurotrophic factor dysfunction has been suggested to play a key role in these disorders (e.g., Castren, 2004, 2005).

Over 19 million adults in the United States alone are affected by anxiety disorders, which cover a range of conditions from generalized anxiety disorder, panic attack to specific phobias (US Department of Health, 2002). Failure to control anxious thoughts or rituals is also central to obsessive-compulsive disorder. Unlike schizophrenia and depression, the opportunity for post-mortem analysis of anxiety in humans is limited by the lack of suitable post-mortem materials. No studies to date have investigated possible relationship between anxiety and brain neurotrophic factors in human. Instead, related studies in human subjects mainly rely on neuro-imaging. Fear inducing stimuli have been reported to generate greater activation of the amygdala and hippocampus in phobic patients (Veltman et al., 2004; Schienle et al., 2005). In Pavlovian fear conditioning, healthy controls also showed enhanced activation in the limbic circuit including the amygdala and

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the hippocampus (Knight et al., 2004; Birbaumer et al., 2005). These findings agree that amygdala and the hippocampus play a key role in the acquisition and expression of emotion and emotionally-laden memory (Davis, 1992; Gray, 1982; LeDoux, 1996; Gray and McNaughton, 2000; Maren, 2001; McGaugh, 2004), and have lent some credence to the suggestion that hippocampal hyperactivity may underlie some forms of anxiety disorders (McNaughton, 1997).

However, there are indications that the relationships between brain regional activation and anxiety/fear response may differ between healthy controls and specific patient groups: fear conditioned stimuli decrease amygdala and hippocampal activation in normal subjects, but enhances activation in both regions among patients with social phobia (Schneider et al., 1999; but also see Rusch et al., 2001). Interestingly, a recent imaging study also revealed similar opposing trends in animals: Kalisch et al. (2005) demonstrated a negative linear relationship between anxiety expression and hippocampal volume in out-bred Wistar rats. The same study also compared rats selectively bred for extremely high against extremely low anxiety phenotype and revealed that the ''high-anxiety related behaviour'' line paradoxically showed an increase in hippocampal volume.

Despite the advent of functional imaging techniques, the study of the patho-physiology of fear and emotion still largely relies on the use of animal models (LeDoux, 1996). Here, we made use of the fact that variability in anxiety-like behaviour can be readily detected in mice using the standard elevated plus maze test of anxiety (Pellow et al., 1985; Pellow and File, 1986) and the Pavlovian tone-shock conditioning paradigm for learned fear (Maren and Quirk, 2004). Indices of fear and anxiety-related behaviour were then correlated with the regional contents of specific neurotrophic factors obtained by post-mortem analysis. We focused on the amygdala and the hippocampus as both are implicated in the regulation and control of anxious response and conditioned fear. In addition, we separately analyzed the dorsal and ventral halves of the hippocampus because of the recent suggestion that the two poles of hippocampus differ in their involvement in emotional behaviour (Kjelstrup et al., 2002; Bannerman et al., 2004; McHugh et al., 2004). The common in-bred mouse strain of C57BL/6 was chosen so as to facilitate comparison with existing data derived from genetic manipulated mouse lines with altered expression of neurotrophic factors (e.g., Croll et al., 1999; MacQueen et al., 2001; Gorski et al., 2003). The use of an in-bred mouse line here also emphasizes the important contribution of epigenetic factors in behavioural variation within a genetically homogeneous population. Subjects of both genders are included to further allow the detection of possible sex differences in behaviour as well as neurotrophic factor content.

The present correlative study is specifically designed to allow the examination of possible associations between postmortem neurotrophic factor levels in the hippocampus and amygdala to individual differences in anxiety/fear behaviour assayed prior to sacrifice. The correlative approach, however, precludes any conclusion as to whether the variations revealed in the postmortem analysis are causally linked to the observed behavioural variations (or vice versa). Nonetheless, the data generated in the present study will permit more specific and focal manipulative experiments to be conducted in the attempt to identify any such potential causal links.

Despite the present focus on fear- and anxiety-related phenotypes, the results would be of relevance to psychiatric conditions with a known high incidence of co-morbidity with anxiety symptoms, including depression (Weissman et al., 1993; Cosoff and Hafner, 1998; Rapaport, 2001), in which a dysfunction in neurotrophic factor signalling, and in particular of BDNF, has been hypothesized (Castren, 2004, 2005).

Methods

Subjects

The subjects were naïve adult C57BL/6J mice obtained from Charles River Laboratories (Germany), originated from The Jackson Laboratory. They were approximately 10–11 weeks old, weighing 25–30 g, at the time of testing. Experiments 1 and 2 employed 16 and 24 mice, respectively, with equal number of male and female mice in each cohort. We designed for a larger sample size for Experiment 2 because we anticipate that behavioural variability might otherwise be limited for the present purpose of seeking statistical correlation between brain neurotrophic factors indices and behaviour. It is because the behaviour of interest in Experiment 2 is learned behaviour induced by means of classical conditioning, whilst that in Experiment 1 represents a form of spontaneous reaction to a given experimental setting. Behavioural testing took place following three weeks of acclimatization to the laboratory animal vivarium (22 ± 2°C, relative humidity at $55 \pm 5\%$, lights on from 20:00 to 08:00). Male and female mice were separately caged in groups of four, and maintained on an ad lib diet throughout the experiment. Testing was always conducted in the dark phase of the cycle. 48 h following the end of the behavioural experiments, the animals were killed by decapitation for post-mortem analysis of brain neurotrophic factor content. All procedures described here had previously been approved by the Cantonal Veterinarian Office of Zurich, in accordance to the European Communities Council Directive – 86/609/EEC.

Apparatus and behavioural procedures

Experiment 1: Elevated plus maze. The apparatus and procedure adopted here have been previously confirmed to be sensitive to anxiolytic drugs (Hagenbuch et al., 2006). The elevated plus maze was made of clear acryl glass, and elevated at a height of 70 cm above floor level. It consisted of four equally spaced arms radiating out from a central square measuring 5×5 cm.

Each arm was 30 cm long. Two opposing arms were enclosed by 15 cm high opaque walls from all sides except the side adjoining the central square. The other two arms were exposed and the outer rim of the open arms was guarded by a perimeter border of 1 mm high. The floor of the entire maze was covered by a grey plastic in-lay that could be easily removed and cleansed with water between trials.

The maze was located in a dimly lit experimental room. The light level in the open arms of the two mazes was balanced at 30 lux. A digital camera was mounted above the maze and images were transmitted at a rate of 5 Hz to a personal computer running the software Ethovision® V2.3/3.0 (Noldus IT, Wageningen, The Netherlands) allowing the tracking of the subject's position. An entry into an open or closed aim was scored when the centre of area crossed the virtual line separating the arm from the central square of the maze.

To begin a trial, the mouse was placed in the centre of the maze with it facing one of the open arms. It was allowed to move freely undisturbed for 5 min before being returned to the home cage.

The mice's reluctance to venture into the exposed open arms was taken as a measure of anxiety. This was indexed by the frequency of arm entries and time spent on the open arms: these measures were expressed as percentage scores over the total number of all (open and enclosed) arm entries, and total time spent in all arms, respectively. In addition, the total distance traversed in the entire maze surface was taken as a measure of general motor activity.

Experiment 2: Conditioned Freezing. Two sets of charmbers were used to provide two distinct contexts. The first set of chambers (context A) comprised two Coulbourn Instruments (P.A., USA) operant chambers (Model E10-10) each installed in a ventilated, sound-insulated chest. The chamber of context A measured $30 \times 25 \times 29$ (high) cm, but the animal was confined to an area of 17.5×13 cm in the center by a transparent Plexiglas enclosure. Illumination inside the chamber was provided by a house light (2.8 W) positioned on the panel wall, 21 cm above the grid floor. The grid floor was made of stainless steel rods (4 mm in diameter) spaced at regular intervals of 10 mm centre to center, and through which scrambled electric foot shock at 1s duration and 0.3 mA intensity (the unconditioned stimulus, US), generated by a shock scrambler (Model E13-14), could be delivered.

The second set of chambers (context B) comprised two cylindrical (19 cm in diameter) enclosures made of clear Plexiglas and painted in light grey, rested on a metal mesh floor; each enclosure was located in a ventilated, sound-insulated, wooden cabinets. It was illuminated by an infra red light source instead of visible light.

All four chambers also contained a sonalert unit (Model H12-02M-2.9), which could deliver a 2.9 kHz tone measuring approximately 86 dB. This provided the conditioned stimulus (CS). In addition, a miniature digital camera was mounted 30 cm directly above the center of the area of interest. The output of the camera was fed to a multiplexer (YSQ-430, Sony, Japan) before being transmitted to a computer (Power Mackintosh 7600/120) running the NIH Image software (version 1.61) for real-time analysis. The algorithm of the freezing response detection procedure has been validated and fully described before (Richmond et al., 1998) and adopted by several other laboratories (e.g., Anagnostaras et al., 2000; Contarino et al., 2002; Huerta et al., 2000; Marchand et al., 2003). Briefly, successive digitized images ($192 \times 144 = 27{,}648$ pixels, at 8-bit grey scale) obtained at a rate of 1 Hz were compared. The number of pixels differed between adjacent frames was then computed. If this was less than 0.05% of the total number of pixels in a frame, the animal was considered to be freezing in that 1-s interval.

On day 1, all animals were given three separate CS-US (tone-shock) pairings, presented at 3-min intervals, in context A. In each pairing, the 1-s shock US followed immediately the 30-s tone CS. On the day of conditioning, the amount of freezing during the three occasions of tone presentation provided a measure for the evaluation of the acquisition of conditioning.

On day 2, the animals were returned to context A. They were placed in the test chamber for a period of 8 min. This served as a test of context freezing. The expression of context freezing was indexed as percent time freezing across the 8 min period.

On days 3–5, CS-freezing to the tone stimulus was assessed in context B. The tone stimulus was administered 3 min after the animals were placed into the test chamber. The tone remained on for a period of 8 min, to parallel the test period of context freezing.

Brain sample preparation and ELISA of neurotrophin content

48 hrs following the completion of the behavioural experiment, the animals were killed by decapitation. The brains were extracted *in toto*, the amygdala and the hippocampus and were then dissected out on an ice-cooled plate. With the brain resting on its dorsal surface on the plate, two 3-mm cuts in the coronal plan were made to delimit the anterior-posterior extent of the amygdala in both hemispheres, extending approximately 2 mm off the mid-line to the lateral extremity. The anterior-posterior placements of these cuts were guided by landmarks (the medial eminence) that are visible on the ventral brain surface in accordance to the Paxinos and Franklin (2003) mouse brain atlas. The cortical mantle lateral and ventral to the amygdala was then excised to expose the amygdaloid complex, which was removed in its entirety without distinction between different anatomically defined nuclei. The two hemispheres were then separated, and the hippocampus extracted *in toto*, including hippocampus proper and dentate gyrus. Each hippocampus was then bisected into two halves of equal length, corresponding to the dorsal (or septal) and ventral (or temporal) hippocampus. Enzyme-linked immunosorbent assay (ELISA) of the content of three neurotrophic factors, BDNF, NGF and NT-3, were carried out under blind condition the following day.

Tissue samples from the left and right hemispheres from individual animals were combined. They were placed in ice-cold Eppendorf tube and homogenized in ice-cold lysis buffer (500 μl per sample), containing 137 mM NaCl, 20 mM Tris–HCL (pH 8.0), 1% NP40 10% glycerol, 1 mM PMSF 10 μg/ml aprotinin, 1 μg/ml leupetin, 50 mM sodium vanadate. Homogenization was achieved in a Dounce homogenizer (10 strokes) followed by sonication at 4°C. The homogenate was then centrifuged $14{,}000 \times g$ for 5 min at 4°C. The supernatnats were collected into separate ice-cold Eppendorf tube and used the determination of total protein and neurotrophin levels.

The total protein level was determined by the use of the BCA Protein Assay Reagent kit (Peirce, Switzerland). BDNF, NGF and NT-3 levels were assessed in selected brain regions using the Promega ELISA assay kit. Briefly, standard 96-well flat-bottom NUNC-Immuno maxisorp ELISA plates were incubated with the corresponding captured antibody, which binds to the neurotrophin of interest, overnight at 4°C. The next day the plates were blocked by incubation for 1 h at room temperature (RT) with a $1 \times$ Block & Sample buffer. Serial dilutions of known amount of NGF and BDNF ranging from 500 to 0 pg/ml were performed in duplicate for the standard curve. Wells containing the standard curves and supernatants of brain tissue homogenates were incubated at RT for 6 or 2 h, as specified by the protocol. They were then incubated with secondary specific antibody overnight at 4°C or for 2 h RT, as specified by the protocol. Next, a species-specific antibody conjugated to horseradish peroxidase (HRP) was used as a trtiary reactant for 2.5 or 1 h at RT. 'TMB One Solution' was used to develop color in the wells. This reaction was terminated with 1N hydrochloric acid at a specific time (10–15 min) at RT, and the absorbance was then recorded at 450 nm in a plate reader within 30 min of stopping the reaction. The neurotrophin values were evaluated by comparison with the regression line for each proposed neurotrophin standard. Using these kits, NGF and BDNF can be quantified in the range of 7.8–500 pg/ml, and NT-3 can be quantified in the range of 4.7–300 pg/ml. For each assay kit, the cross-reactivity with other trophic proteins is $\leq$2–3%

Statistical analysis

All analyses were conducted using the statistical software SPSS for Windows (version 13) implemented on a PC running the Windows XP

(SP2) operating system. The ELISA and behavioural data sets were first separately subjected to parametric analyses using: analysis of variances (ANOVA) with the between-subjects factor sex (male vs. female), and then subjected to bivariate correlative analysis.

To directly compare the levels of neurotrophic factors expression between the two halves (dorsal vs. ventral) of hippocampus, the hippocampal measures obtained were subjected to a split-plot ANOVA with the additional within-subject factor of septo-temporal axis. Correlation between different region-specific neurotrophic factors contents obtained in each experiment was also evaluated independently by correlative analyses.

Behavioural data of Experiment 1 were analysed by one-way ANOVA with the between-subjects factor sex (male vs. female). Analysis of behavioural data from Experiment 2 required the additional within-subject factor of days or trials whenever appropriate using a split-plot ANOVA design.

Association between region-specific neurotrophic factor content and behavioural data was examined by Pearson's product moment correlation first by combining both male and female data. Significant correlation revealed was further subjected to partial correlation analyses to identify if the correlation might solely reflect concomitant sex-dependent effects on both the two variables being subjecte do correlative analysis. Additional correlative analyses restricted to either sex were also conducted when we wish to examine the extent to which the signification correlation revealed was equivalently seen in both sexes.

A p-value of <0.05 at the two-tailed criterion is taken as statistically significant.

Results

Neurotrophic factor content

Experiment 1

Amygdala. Separate one-way ANOVA revealed a significant sex difference in NGF content with male mice showing a higher level [$F(1, 15) = 9.15$, $p < 0.01$]. No sex difference was apparent in BDNF or NT-3 content (see Fig. 1A–C).

Hippocampus. Separate 2-way (sex × septo-temporal axis) split-plot ANOVAs were conducted to assess the differential expression of the three neurotrophic factors

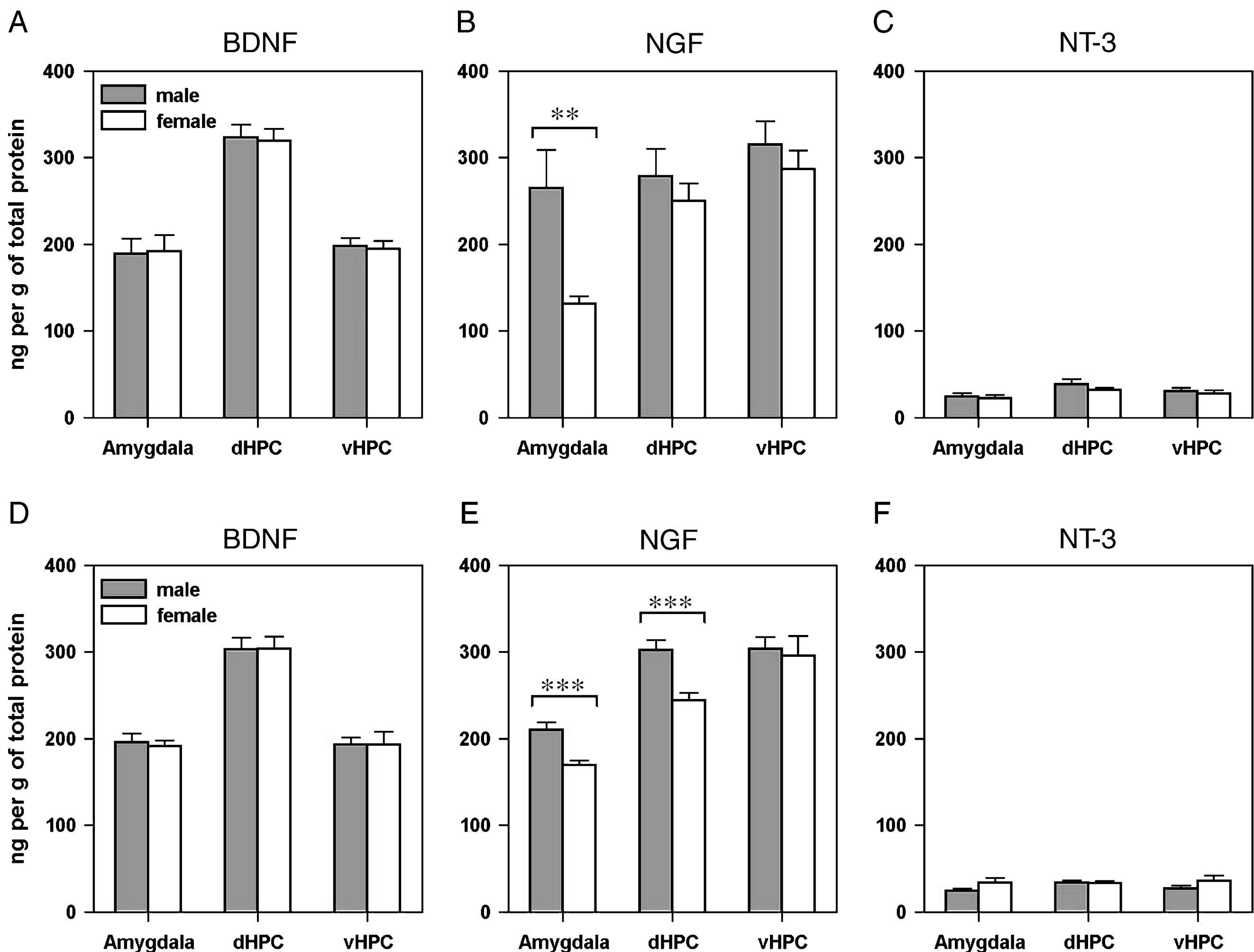

Fig. 1. Mean content of three neurotrophic factors (BDNF, NGF and NT-3) in the amygdala, dorsal hippocampus (dHPC) and ventral hippocampus (vHPC) obtained in Experiment 1 (A, B, C) and Experiment 2 (D, E, F), expressed as a function of sex. **Indicates significant sex difference at $p < 0.01$, and *** at $p < 0.001$. Error bars refer to +SEM of the corresponding mean values

between dorsal and ventral hippocampus. The analyses revealed a significant dorso-ventral difference in BDNF content [$F(1, 14) = 108.25$, $p < 0.001$], but not in NGF [$F(1, 14) = 3.43$, $p = 0.09$] or NT3 [$F(1, 14) = 4.07$, $p = 0.07$] levels. The content of BDNF in the dorsal hippocampus was approximately 64% higher than in the ventral hippocampus. No sex difference was revealed either as a main effect [all F's < 1] or as an interaction with the factor septo-temporal axis [all F's < 1], suggesting that the general dorso-ventral pattern revealed here did not differ between sexes.

Experiment 2

Amygdala. A sex difference was again revealed in the NGF content [$F(1, 22) = 15.51$, $p < 0.001$] in the same direction as in Experiment 1 (see Fig. 1E). Neither BDNF [$F < 1$] nor NT-3 [$F(1, 22) = 2.90$, $p = 0.10$] levels showed any significant difference between sexes (see Fig. 1D and 1F). The findings are therefore consistent with those of Experiment 1.

Hippocampus. A clear dorso-ventral difference in BDNF content was observed [$F(1, 22) = 81.86$, $p < 0.001$], with dorsal BDNF content 60% higher than the ventral level. The results are highly comparable with those obtained in Experiment 1 in both absolute and relative terms. Again, this contrasted with the lack of a significant dorso-ventral difference in NGF [$F(1, 22) = 3.45$, $p = 0.08$] and NT-3 [$F < 1$] content.

The analysis further revealed a sex effect specifically for NGF content [$F(1, 22) = 4.38$, $p < 0.05$] that was not seen in Experiment 1. Male mice showed a higher level of NGF, and this was particularly pronounced in the dorsal hippocampus. Additional analysis restricted to dorsal NGF content indicated a significant difference [$F(1, 22) = 16.80$, $p < 0.001$]; a restricted analysis to ventral NGF content in contrast did not yield a significant sex difference [$F < 1$]. However, interpretation of this contrast should be cautious because: (i) the lack of a significant sex $\times$ septo-temporal axis interaction [$F(1, 22) = 3.07$, $p = 0.09$] in the overall analysis here, and (ii) a similar tendency for such a gender-dependent septo-temporal difference in hippocampal NGF content was lacking in Experiment 1.

No sex difference was observed in the BDNF or NT-3 content either as a main effect [all F's < 1] or as an interaction with the factor septo-temporal axis [maximum $p = 0.2$].

Taken together, data from both experiments strongly support the presence of a pronounced dorso-ventral difference in hippocampal BDNF content. No significant dorso-ventral difference in hippocampal NGF content was shown in both experiments, and this was in spite of the increase of sample size in Experiment 2. Notably, the non-significant dorso-ventral difference of NGF content was in opposite direction to that demonstrated for hippocampal BDNF content (also see correlative analysis below).

There was a clear absence of any sex difference in hippocampal BDNF or NT-3 content. A significant sex difference in hippocampal NGF was detected in Experiment 1 but not Experiment 2. Given the difference in sample size, the presence of a similar trend in both experiments, and the reduced standard error of the means in Experiment 2 in comparison to Experiment 1, it is likely that the presence and absence of the sex difference in hippocampal NGF reflects a difference in statistical power rather than the fact that the two cohorts of animals had underwent distinct behavioural manipulations prior to sacrifice.

Correlation among region-specific neurotrophic factors levels

Additional analyses were conducted to examine possible association among the nine neurotrophic factor measures. To this end, data from the two experiments were combined. We adopted this strategy to strengthen statistical power, and because most of the neurotrophic factor measures did not differ between sexes (see above). We also performed additional analyses to compare directly the nine measures obtained in the two experiments. None of these yielded a significant between-experiments difference (with p-values ranging from 0.17 to 0.8).

The results of Pearson's correlative analysis and partial correlation controlling for between-sex and between-experiment variation are illustrated in Table 1A and B, respectively. Ten significant associations were identified to be consistently significant in both analyses. Eight of them involve NT-3 levels. One was between ventral hippocampal BDNF and ventral hippocampal NGF levels. One was between dorsal hippocampal NGF and amygdale NGF levels.

Elevated plus maze test of anxiety

Behaviour

Two measures of anxiety-related behaviour (the percent frequency of arm entries into open arms, and the percent time spent on the open arms) and one measure of general locomotor activity (total distance traversed) were subjected to a one-way ANOVA with the between-subjects factor of sex. None of the comparisons yielded any significant sex difference. The results are summarized in Table 2.

Table 1. *Correlation matrix illustrating association among the nine different region-specific neurotrophic factor measures obtained across the two experiments. (A) Correlation coefficients (with corresponding p-value illustrated below) derived from bivariate Pearson's product moment correlation. (B) Correlation coefficients derived from partial correlation analyses with the factors sex and experiment controlled for. Significant association is indicated by grey boxes. Correlation that only attains statistical significance in one or the other matrix is highlighted by italics. The ten correlations that are significant in both analyses are indicated by bold borders in (B)*

(A) Correlation (df=40-2=38)

		BDNF			NGF			NT-3		
		Amy	dHPC	vHPC	Amy	dHPC	vHPC	Amy	dHPC	vHPC
BDNF	Amy	1	0.02 0.902	0.13 0.437	0.11 0.511	0.07 0.649	-0.20 0.207	**0.36 0.023**	0.24 0.144	0.03 0.831
	dHPC		1	0.08 0.622	-0.17 0.306	-0.18 0.259	0.01 0.928	-0.09 0.592	0.00 0.987	-0.28 0.079
	vHPC			1	-0.09 0.594	0.07 0.676	**0.43 0.005**	**0.35 0.025**	0.26 0.107	**0.48 0.002**
NGF	Amy				1	**0.63 0.000**	0.19 0.240	0.05 0.782	**0.38 0.017**	0.05 0.760
	dHPC					1	0.28 0.085	-0.02 0.921	**0.46 0.003**	0.03 0.843
	vHPC						1	0.27 0.096	*0.32 0.046*	**0.61 0.000**
NT-3	Amy							1	*0.27 0.092*	**0.50 0.001**
	dHPC								1	**0.33 0.039**
	vHPC									1

(B) Partial Correlation controlling for Sex & Experiment (df=40-2-1-1=36)

		BDNF			NGF			NT-3		
		Amy	dHPC	vHPC	Amy	dHPC	vHPC	Amy	dHPC	vHPC
BDNF	Amy	1	0.03 0.863	0.13 0.444	0.12 0.487	0.07 0.684	-0.21 0.210	**0.37 0.023**	0.24 0.148	0.03 0.835
	dHPC		1	0.07 0.660	-0.22 0.176	-0.19 0.247	0.01 0.946	-0.04 0.793	-0.02 0.921	-0.27 0.098
	vHPC			1	-0.12 0.480	0.07 0.675	**0.43 0.007**	**0.38 0.018**	0.26 0.120	**0.49 0.002**
NGF	Amy				1	**0.54 0.000**	0.14 0.386	0.20 0.235	**0.35 0.031**	0.15 0.371
	dHPC					1	0.25 0.132	0.05 0.772	**0.45 0.004**	0.09 0.605
	vHPC						1	0.31 0.060	*0.30 0.064*	**0.64 0.000**
NT-3	Amy							1	*0.34 0.040*	**0.49 0.002**
	dHPC								1	**0.36 0.025**
	vHPC									1

Table 2. *Summary of the three behavioural measures obtained in Experiment 1 between male and female subjects, which never differed significantly from each other*

	Male	Female
Percent frequency into open arms (%)	4.8 ± 1.8	3.1 ± 0.9
Percent time in open arms (%)	15.2 ± 4.7	12.8 ± 3.5
Total distance (cm)	813.2 ± 31.6	729.1 ± 54.5

Correlation between behavioural indices and neurotrophic factor levels

Next correlative analyses were conducted to examine possible relationships between the behavioural and ELISA measures obtained in Experiment 1, and the results are summarized in Table 3. This revealed six significant correlations. Table 3 also illustrates that these correlations remain statistically significant in a partial correlation controlling for sex differences, indicating that observed associations cannot be solely accounted for by variation attributable to variation due to sex.

BDNF content in the dorsal hippocampus correlated negatively with the proportion time and entries into open arms [both p's < 0.001]. This suggested a positive relationship with anxiety-related behaviour: a higher level of dorsal hippocampus BDNF was associated with increased reluc-

Table 3. *Pearson's product moment correlation coefficients obtained from correlations between regionally specific neurotrophic factor (BDNF, NGF and NT-3) contents and behavioural measures from the elevated plus maze experiment (Experiment 1). Correlative analyses were performed collapsed across sexes (male = 8, female = 8, df = 14). For the significant correlations, results from additional partial correlation, controlling for the factor sex, are given in parenthesis (df = 13). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. The two anxiety indices measure the reluctance of the subjects to venture into the open arms, hence a numerically negative correlation here refers to a positive association between the neurotrophic factor and anxiety*

	BDNF			NGF			NT-3		
	Amygdala	dHPC	vHPC	Amygdala	dHPC	vHPC	Amygdala	dHPC	vHPC
% open arms time	−0.05	-0.78^{***} (-0.79^{***})	−0.20	$+0.54^{*}$ $(+0.61^{*})$	$+0.63^{**}$ $(+0.63^{**})$	−0.02	+0.29	+0.36	+0.38
% open arms entries	−0.09	-0.76^{***} (-0.79^{***})	−0.23	$+0.58^{*}$ $(+0.61^{*})$	+0.44	+0.00	+0.30	+0.44	+0.48
Total distance	−0.06	−0.23	−0.20	$+0.50^{*}$ (+0.40)	+0.29	−0.08	+0.13	+0.11	+0.07

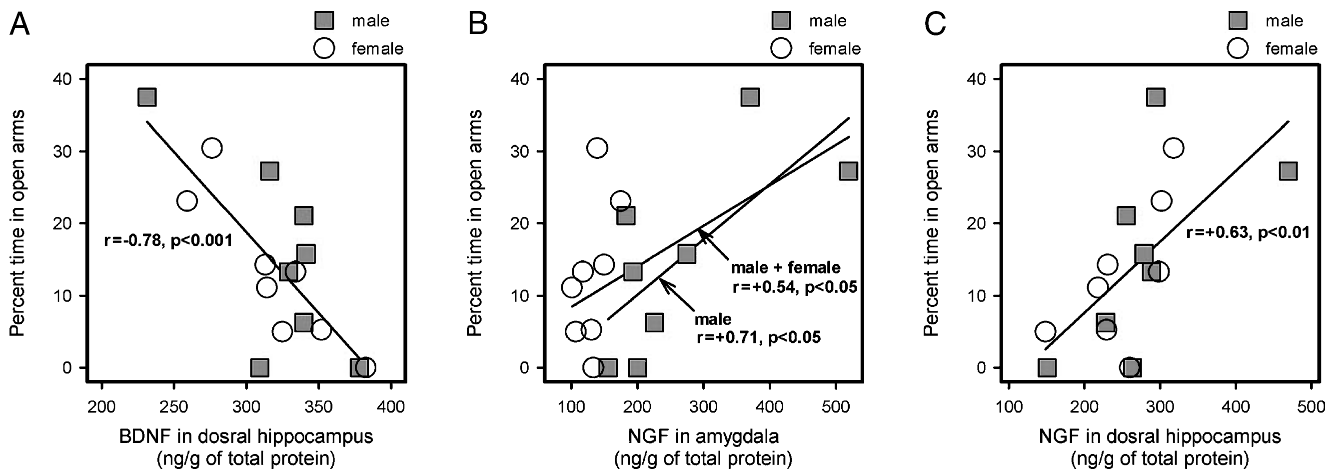

Fig. 2. Scatter plots illustrating the relationship between anxiety-related behaviour (proportion time spent in open-arms) and three regional specific measures of neurotrophic factors: (A) BDNF content in dorsal hippocampus, (B) NGF content in the amygdala, or (C) NGF content in dorsal hippocampus. The solid line in each plot represents the fitted linear regression line (all p's < 0.05). See Table 3 for levels of statistical significance. Data points derived from male and female subjects are distinguished by square and circle symbols, respectively. Correlation in the female alone of the data illustrated in (B) did not attain statistical significance and the corresponding regression line is not illustrated

tance to venture into the open arms of the elevated plus maze (see Fig. 2A). Notably, partial correlation conducted to control for the influence of sex difference confirmed that the observed association remained statistically significant.

On the other hand, NGF levels in the amygdala was related positively to the proportion time and entries into open arms [both p's < 0.05]. Thus, higher amygdala NGF content was associated with reduced sign of anxiety (see Fig. 2B). Partial correlation suggested that these associations did not stem from concomitant covariance attributable to sex differences. However, as illustrated in Fig. 2B, the correlation was only clearly seen in the male mice, and this impression is confirmed by separate correlative analyses restricted to either male [$r = +0.71$, $p < 0.05$, df $= 6$] or female mice [$r = +0.50$, $p < 0.21$, df $= 6$]. These additional analyses were appropriate given the presence of a significant sex difference in amygdala NGF levels.

A positive association between amygdala NGF levels and general locomotor activity was also revealed [p's $<$ 0.05]. This association was no longer statistically significant when the variation due to sex was controlled for using a partial correlation. Separate correlative analyses indicated the lack of significance when the data set was restricted to either male [$r = +0.69$, $p = 0.06$, df $= 6$] or female mice [$r = +0.37$, $p = 0.37$, df $= 6$]. These results confirmed that the overall correlation between amygdala NGF levels and locomotor activity was solely attributed to sex differences in both the behavioural and protein measures. The significant association between dorsal hippocampal NGF levels and anxiety [$p < 0.01$] observed was opposite in direction to that seen between amygdala NGF level and anxiety. Statistical significance of the former was however only seen in the measure of percent time in open arms (see Fig. 2C) but not the other behavioural index of anxiety.

Notably, NT-3 content did not significantly correlate with any behaviour measures obtained in the elevated plus maze.

Pavlovian conditioned freezing

Behaviour

Acquisition of conditioned freezing was assessed on the first day of testing across the three trials of tone-shock pairings (see Fig. 3A). The amount of freezing (in percent time) was submitted to a 2×3 (sex $\times$ trials) split-plot ANOVA. This revealed a significant main effect of trials [$F(2, 44) = 76.56$, $p < 0.001$], indicative of increasing amount of freezing over trials, and a significant main effect of sex [$F(1, 22) = 4.73$, $p < 0.05$] with the male mice exhibiting a higher level of freezing. There was no evidence for a difference between the two genders in the rate of increase in freezing across trials [sex $\times$ trials interaction: $F(2, 44) = 1.84$, $p = 0.17$].

Context freezing was measured on the second day of testing when the animals were returned to the shocked context (see Fig. 3B). A 2×8 (sex $\times$ 1-min bins) split-plot ANOVA did not reveal any significant effect.

Next, the expression of conditioned freezing to the tone and its extinction across days were examined across days 3–5, when the tone CS was presented in a context distinct from the one in which conditioning had taken place (see Fig. 3C). On each CS-test day, the tone was presented for a period of 8 min, and the dependent measure of percent time freezing was subjected to a $2 \times 3 \times 8$ (sex $\times$ days $\times$ 1-min

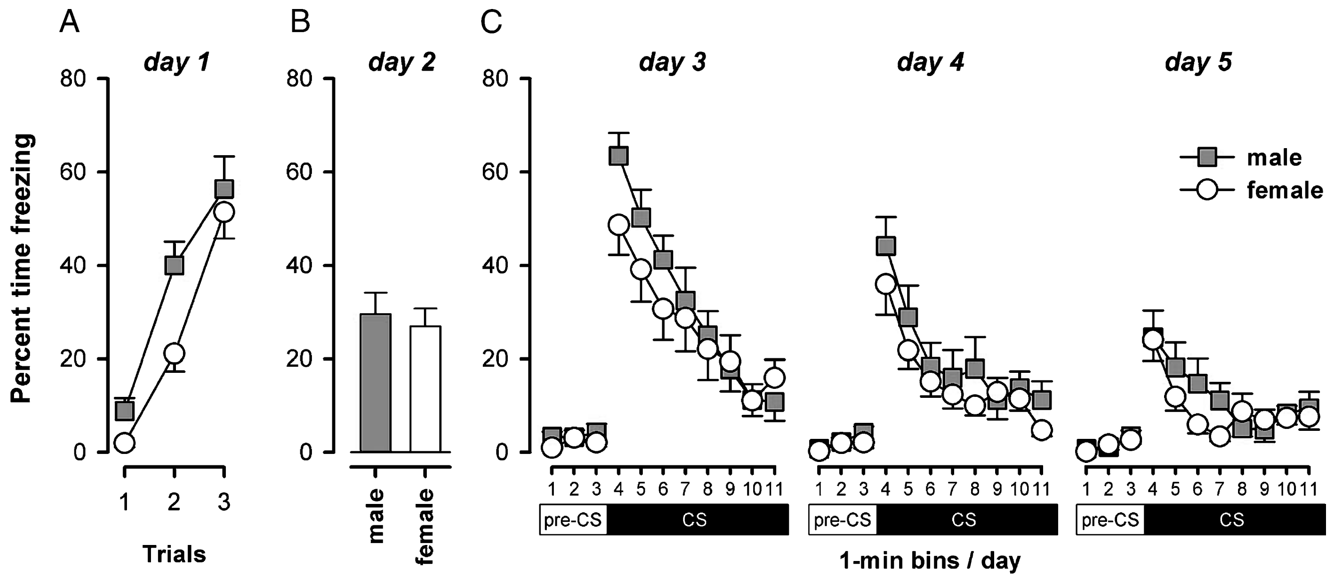

Fig. 3. Behavioural measures obtained from the conditioned freezing experiment (Experiment 2). (A): percent time freezing in the presence of the tone CS (30 s in duration) expressed as a function of conditioning trials on day 1. (B): percent time freezing to the context on day 2 (8 min in duration). (C): percent time freezing across three tone-test days (days 3–5), with percent time freezing expressed as a function of successive 1-minute bins: no CS was present in the first 3 min ('pre-CS' period), CS was presented in the next 8 minutes ('CS' period). All values refer to mean ± SEM

bins) split-plot ANOVA. The analysis yielded a significant effect of days [F(2, 44) = 32.88, $p < 0.001$], of bins [F(77, 154) = 49.60, $p < 0.001$], and of their interaction [F(14, 308) = 8.77, $p < 0.001$]. The interaction term reflects that within-session extinction was most apparent on the first CS-test day. There was no evidence for any significant sex difference in the absolute level of conditioned freezing and its reduction over days. In the CS-tone test, neither the main effect of sex nor its interaction attained statistical significance.

Correlation between behavioural indices and neurotrophic factor levels

Three indices of conditioned freezing were selected for the purpose of correlative analysis. They were (a) the mean percent time freezing across the three CS presentations on day 1, (b) the mean percent time freezing to the context on day 2, and (c) the mean percent time freezing across the three days of tone-freezing test across days 3–5. The levels of tone freezing across the three days were highly inter-correlated with each other ($r = +0.72$ to $+0.88$, df = 46, $p < 0.0001$], and they were therefore pooled to form a single measure. The results are summarized in Table 4. NGF content in the amygdala ($p < 0.05$) and in the dorsal hippocampus ($p < 0.01$) correlated positively with the levels of tone-freezing observed on day 1 (see Fig. 4B and C). These two variables, however, did not correlate with tone-freezing across days 3–5. Instead, BDNF content in the amygdala was observed to correlate positively ($p < 0.05$) with tone-freezing across days 3–5 (see Fig. 4A). No other significant correlation was obtained.

Table 4. *Pearson's product moment correlation coefficients obtained from correlations between regionally specific neurotrophic factor (BDNF, NGF and NT-3) contents and behavioural measures from the conditioned freezing experiment (Experiment 2).* $^{*}p < 0.05$, *df = 22. For the significant correlations, results from additional partial correlation, controlling for the factor sex, are given in parenthesis (df = 21).* [a,b]*Partial correlative analyses indicated that the two significant correlations no longer attained significance when the effects of sex were controlled for [p = 0.33, and p = 0.14, respectively]*

	BDNF			NGF			NT-3		
	Amygdala	dHPC	vHPC	Amygdala	dHPC	vHPC	Amygdala	dHPC	vHPC
Day1: CS-freezing	+0.26	+0.35	+0.14	+0.42* (+0.21[a])	+0.49* (+0.32[b])	−0.13	−0.16	+0.34	−0.23
Day 2: Context-freezing	+0.26	+0.26	+0.20	+0.02	+0.29	−0.09	−0.18	+0.32	−0.08
Days 3–5: CS-freezing	+0.43* (+0.43*)	+0.23	+0.23	+0.15	+0.34	+0.05	−0.10	+0.15	0.02

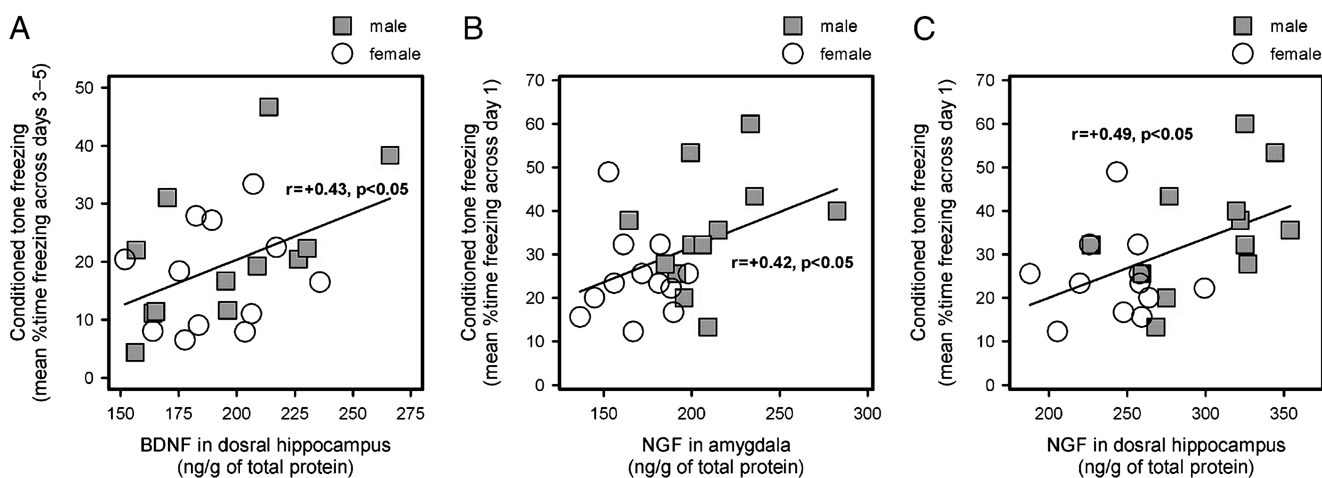

Fig. 4. (A): Scatter plots of the measure of conditioned freezing (percent time freezing) across the three tone-CS test across days 3–5 versus amygdala BDNF protein content. (B) and (C): Scattered plots of the measure of conditioned freezing (percent time freezing) to the tone CS across the three conditioning trials on day 1 versus NGF protein content in the amygdala, and that in the dorsal hippocampus, respectively. The solid line in each plot represents the fitted linear regression line (all p's < 0.05). See Table 4 for levels of statistical significance. Data points derived from male and female subjects are distinguished by square and circle symbols, respectively. Partial correlation controlling for the factor sex indicated that the overall significant correlation illustrated in (B) and (C) did not reach statistical significance (see Table 4)

Out of the three correlations attaining statistical significance, two (depicted in Fig. 4B and C) would have been anticipated by the concomitant significant sex difference in the predictor variables (amygdala NGF, and hippocampal NGF content) and the predicted variable (tone-freezing during conditioning on day 1). Additional partial correlative analyses were therefore conducted to assess the contribution of the factor sex to the additional partial correlative analysis. When the effects of sex were controlled for, the partial correlation between tone-freezing on day 1 and the two NGF measures no longer achieved statistical significance ($p = 0.14$–0.33). These results suggested that the correlations relating CS-freezing on the conditioning day to post-mortem NGF levels in amygdala and dHPC are attributed solely to sex differences in both the behavioural and protein measures. Consistent with this impression, correlations restricted to either sex failed to attain statistical significance.

Discussion

Sex differences

The inclusion of subjects of both genders here has allowed us to examine the possible presence of sex differences in all dependent measures. First, no sex difference in anxiety behaviour was revealed in Experiment 1, and a sex difference in freezing behaviour was only seen on the conditioning phase of Experiment 2. Second, the levels of BDNF were remarkably similar between sexes in all three brain regions examined, and this is a consistent observation in both experiments (Fig. 1A and D). This contrasts with evidence that brain BDNF gene expression shows sexual dimorphism in rats (Bland et al., 2005) and in zebra finches (Dittrich et al., 1995), and that gender differences have been noted in treatment-induced BDNF gene expression (e.g., Matsuki et al., 2001). Third, a similar lack of sex difference was also seen in the NT-3 levels across the three brain regions; and NT-3 content is relatively low in comparison to that of BDNF and NGF; this is in keeping with previous data collected in C57BL6 mice in our laboratory (see Zhu et al., 2006). Fourth, a clear sex difference only emerged in the NGF content, which was most clearly and consistently seen in the amygdala. Given that NGF is known to facilitate neurotransmission between nucleus basalis and the amygdala in rats (Moises et al., 1995), our novel demonstration of a robust sexual dimorphism in amygdala NGF content, replicating our previous report (Zhu et al., 2006), clearly warrants further investigation. The sex difference in dorsal hippocampal NGF levels was not statistically significant in Experiment 1, but attained significance in Experiment 2 with an increased sample size. In both cases, the direction of the difference is in line with a previous report showing higher NGF levels in the male (Katoh-Semba et al., 1989). In the rat, a sex difference in hippocampal NGF mRNA expression is only apparent in neonatal subjects (Komack et al., 1991) but not in adulthood (Nishizuka et al., 1991). However, it should be emphasized that the difference we reported here was based on NGF protein levels, and the significant sex

difference revealed in the hippocampus was restricted to the dorsal half.

The correlative approach and interpretative caveat

Our attempt to relate behaviour with brain neurotrophins yielded some support for the speculation that individual differences in the post-mortem levels of BDNF and NGF in dorsal hippocampus and amygdala are functionally related to the observed variability in anxiety-related behaviour among normal mice. At the same time, individual variation in NT-3 bears no relationship with behaviour in the elevated plus maze or in Pavlovian conditioned fear. The use of an inbred mouse strain here suggests that epigenetic factors may underlie the identified brain-behaviour correlation or associations. It has been suggested that manipulation of social interaction and con-specific aggression can lead to brain changes that may give rise to depressive traits (Tsankova et al., 2006 and Berton et al., 2006). This particular factor, however, may be especially relevant to correlations that are only clearly seen in male mice, but not in female mice. Unfortunately, we have not recorded social ranking within cage, and therefore cannot directly assess the extent to which the neurotrophic factors measures and behavioural indices here are further related to home cage social status and/or con-specific social interaction.

Correlative analysis provides evidence for association, but itself does not constitute direct evidence for a causative link. Any interpretation of causal directions, on the basis of the present data alone, is necessarily speculative in nature. At least two interpretations of our findings may be offered here.

First, if the variation in neurotrophic factor levels reflects pre-existing variation prior to the testing phase, then specific individual differences in the regional neurotrophic factors content would be predictive of individual response on the elevated plus maze test of anxiety and the acquisition/expression of conditioned fear. This does not require an assumption that the absolute levels of neurotrophic factors determined 48 h following behavioural testing remain identical to the baseline levels (i.e., levels of, up to that time, behavioural naïve subjects). In spite of the lack of a naïve control group in the present study, which would have been instrumental for the assessment of whether the mean levels of neurotrophic factors were affected by the behavioural procedures as such, the absolute levels of all nine neurotrophic factors measures never differed significantly between the two experiments as supported by direct statistical comparisons. Indeed, the entire profile was remarkably similar between the two experiments. If our behavioural procedures had led to long-lasting and sustained changes in the overall levels of brain neurotrophic factors, some differences would be expected to emerge between the two experiments, which differed greatly not only in terms of test duration, but also the use of stressors or aversive stimuli. Hence, it may be reasonable to speculate that the post-mortem levels assessed at 48 h following behavioural manipulations are likely to reflect baseline levels rather than possible changes shortly after or during the test (see Hall et al., 2000; Rattiner et al., 2004). Furthermore, the pattern and levels of brain neurotrophins reported here did not differ greatly from our published results obtained in behaviourally naïve mice of the same strain (Zhu et al., 2006). While Zhu et al. (2006) showed that experience of multiple behavioural testing can lead to alterations in neurotrophic factor levels in comparison to undisturbed control mice, the direction of such effects are far from simple. No simple main effect of 'naivety' was revealed in amygdala NGF or BDNF levels, and hippocampal NGF levels. Instead, complex interaction between naivety, sex and housing condition are noted (see Table 2 of Zhu et al., 2006). Even when a main effect of naivety emerged with respect to amygdala BDNF levels, it was also accompanied by an interaction with sex.

Second, the variation in post-mortem neurotrophic factors content may be the consequence of the differential response in the respective behavioural tests. This interpretation does not make any reference to the pre-test neurotrophic levels. Instead, this view implies that changes in the observed individual variation in neurotrophic factors accompanied, or developed as a consequence of, the animals' differential response when confronted with an anxiogenic situation. One possibility is that neurotrophic factors signalling is involved in the physiological response repertoire under various anxiogenic situations. If so, such anxiety-dependent variation of neurotrophic factors must have lasted for at least 48 h, and therefore distinct from the behaviour-dependent changes in neurotrophic factors expression reported by Hall et al. (2000) and Rattiner et al. (2004), which appear to be relatively rapid. This view is independent of whether there are overall changes in the mean levels of neurotrophic factors in the tested subjects in comparison to naïve animals, because this interpretation focuses on the expression of the response (towards anxiogenic or anxiolytic direction with respect to the population average). This would not directly predict a change in the mean levels of neurotrophic factor following behavioural tests. Instead it would predict higher inter-individuals variability in the levels of neurotrophic factors in tested subjects in comparison to a sample of naïve subjects.

The present data set cannot decide between the two interpretations above, and additional experiments will be required to distinguish between the two causal interpretations. Accurate determination of neurotrophic factor content in behaving animals is currently not feasible. A correlative approach therefore cannot relate causally pretest neurotrophic factor levels to individual behavioural differences. To directly test this hypothesis, a manipulative approach using region-specific manipulations of neurotrophic factor would be required. This may be achieved by of intracerebral infusion of BDNF or NGF, their antisense/antibodies, or by means of viral mediated genomic or proteinomic alterations.

Behavioural correlates of BDNF content

Specifically, our data suggest that while dorsal hippocampal BDNF levels are positively related to anxiety-like behaviour observed in the elevated plus maze, amygdala BDNF levels are positively related to the acquisition of conditioned fear to a discrete tone. Hence, BDNF signalling may be related to fear expression in unconditioned (or ethological) as well as conditioned tests of anxiety, and it may do so via multiple brain structures with distinct functional relevance to fear and anxiety (Menard and Treit, 1999; McHugh et al., 2004).

The link between amygdala BDNF protein levels and conditioned tone freezing recorded in the retention test (Fig. 4A) supports the recent finding that amygdala BDNF signalling is involved in the Pavlovian conditioned fear (Rattiner et al., 2004a, b, 2005). In particular, these authors showed that amygdala BDNF mRNA level is elevated following conditioning, which peaks at 2 h post-conditioning. Here, BDNF was measured in mice 6 days following conditioning, so it is unlikely that the correlation observed was induced by the conditioning event. This is strengthened by the fact that the brain levels of BDNF (and NGF as well) obtained in Experiment 2 did not differ from those derived from subjects in Experiment 1, which did not undergo any tone-shock conditioning. Hence, our finding indicates the possibility that higher amygdala BDNF content is associated with the development or expression of a stronger CS-US association. Rattiner et al. (2004a) also provided evidence that amygdala trk-B receptor activation is necessary for normal acquisition of conditioned fear, but it is not required for its expression. This is somewhat in contrast to our observation that a correlation was only apparent in the retention/expression test of conditioned fear, but not in the freezing performance recorded during conditioning.

The direction of the correlation between hippocampal BDNF levels and elevated plus maze anxiety behaviour is in line with our recent observation that housing in enriched environment, which can enhance hippocampal (but not amygdala) BDNF levels, increases anxiety in the same test (Zhu et al., 2006). Of relevance to the present discussion, another study from our laboratory showed that rearing in enriched environment exerted little influence on the acquisition of conditioned freezing (Pietropaulo et al., 2006 – in press), and this is in agreement with the lack of a correlation between dorsal hippocampal BDNF levels and conditioned freezing here.

Approximately 60% of the variance in plus maze anxiety-related behaviour can be accounted for by individual differences in dorsal hippocampal BDNF levels. This high degree of correlation is within the range of correlation reported between hippocampal volume and anxiety score in healthy subjects (≈74%) and amongst depressive patients (≈46%) (Rusch et al., 2001; but see also Kalisch et al., 2005). In contrast, BDNF levels in the ventral hippocampus appear far less important. Moreover, dorsal hippocampal BDNF levels are not related to locomotor activity. On the other hand, amygdala NGF correlated with anxiety indices as well as locomotor activity in the elevated plus maze (see Table 3); we therefore cannot exclude the possibility that the apparent relationship between amygdala NGF and elevated plus maze anxiety behaviour might be mediated via non-specific locomotor effects.

The relevance of hippocampal BDNF to elevated plus maze behaviour, and in particular the emphasis on the dorsal hippocampus, may be surprising to some. First, it is at odds with recent studies showing that disruption of the BDNF gene fails to affect anxiety (MacQueen et al., 2001; Gorski et al., 2003). One possibility is that compensatory mechanisms during development might have masked the regulatory role of hippocampal BDNF signalling in anxiety-related behaviour that is normally present in wild-type animals. Second, the selective involvement of dorsal hippocampus seems contrary to the hypothesis that ventral hippocampus is preferentially involved in anxiety-related behaviour in the rat (Kjelstrup et al., 2003; Bannerman et al., 2004; McHugh et al., 2004). The basal level of BDNF is typically twice as abundant in the dorsal in comparison to the ventral hippocampus (an observation that we repeatedly demonstrated in mice and rats, e.g., Zhu et al., 2006). It is possible that BDNF may normally exert a tighter control over dorsal hippocampal activity. Third, it has been proposed that the therapeutic action of antidepressants involves activation of BDNF-mediated signalling mechanisms (Chen et al., 2001; Castren, 2004). Given that there is

a high degree of co-morbidity between anxiety and depression (Weissman et al., 1993; Rapaport, 2001), and that some anxiety-related disorders such as obsessive compulsive disorders and eating disorders are also treated with antidepressant drugs, one may speculate that increases in BDNF-mediated signalling would be anxiolytic rather than anxiogenic in nature. Additional measures allowing the distinction between extracellular and intracellular BDNF content, quantification at the mRNA levels, the differential utilization of multiple BDNF promoters, as well as trk-B receptor binding studies would be required to support any further interpretation of the present finding, and its integration with data derived from experimental interventions known to affect the BDNF brain content and signalling mechanism.

Behavioural correlates of NGF content

Amygdala NFG content is associated with freezing behaviour recorded across successive CS presentations during conditioning (Fig. 4B), and this is possibly a relationship induced by sex. One speculation is that sex difference in amygdala NGF levels may account for the sex difference seen in the acquisition of Pavlovian conditioned fear. A similar impression also emerges with respect to dorsal hippocampal NGF levels (Fig. 4C). Our findings are thus in line with several reports indicating that (i) fear conditioning involves the coordination of several limbic structures including the amygdala and hippocampus (Bannerman et al., 2004; Maren and Quirk, 2004), (ii) NGF exerts influences on the growth, maintenance and functioning of the cholinergic projection amygdala receives from the nucleus basalis (Moises et al., 1995) as well as the septohippocampal cholinergic system in intact animals (e.g., Fusco et al., 1989).

Notably, NGF levels in amygdala and in the dorsal hippocampus are related to anxiety/fear behaviour in both experiments, with a comparable magnitude of correlation and dependency on sex. However, it should be emphasized that the direction of the relationship observed between NGF levels and the expression of anxiety or fear behaviour in the two experiments are opposite to each other. While NGF levels in hippocampus/amygdala correlated negatively with expressed anxiety levels in the elevated plus maze (Fig. 2B, C), they correlated positively with the acquisition of conditioned fear in the conditioning phase (Fig. 4B, C). It is unlikely that this divergence stems from an underlying relationship with locomotor activity, because while amygdala NGF content is related to activity in the elevated plus maze (Table 3), dorsal hippocampal NGF content is not. Secondly, the correlation observed in the conditioned freezing experiment is specific to the CS-test. If increasing NGF content is non-specifically related to higher general motor activity, then a correlation would be expected across all days in Experiment 2.

NGF is closely related to the cholinergic system of the CNS (see Rattray, 2001). Besides its effects on the differentiation, survival and maintenance of cholinergic neurons in the hippocampus, NGF also exerts a positive influence on cholinergic neurotransmission. Hence, the correlation between dorsal hippocampal NGF and elevated plus maze anxiety might be anticipated by a number of psychopharmacological studies indicating an involvement of the hippocampal cholinergic system in the modulation of anxiety behaviour. Enhancement of hippocampal cholinergic transmission is accompanied by anxiolytic effects, whilst blockade of cholinergic (muscarinic M1) receptors has anxiogenic properties (File et al., 1998; Degroot et al., 2001; Degroot and Treit, 2002, 2003). Variation in NGF content in the amygdala may also reflect differences in cholinergic transmission (Moises et al., 1995). Indeed, the observation that amygdala NGF levels correlated with locomotor activity agrees with an inter-strains comparison in mice identifying a positive effect of muscarinic M1 receptors activation on general activity (Yilmazer-Hanke et al., 2003). The selective involvement of the dorsal (but not the ventral) hippocampus is similar to the correlation observed between hippocampal BDNF and elevated plus maze anxiety. This preferential involvement supports the notion of a functional segregation between the two poles of the hippocampus, but disagrees with the hypothesis derived mainly from selective lesions studies that the ventral instead of the dorsal pole is selectively involved in the processing of anxiety and fear (see Bannerman et al., 2004). The interesting possibility that aberrant fear-related behaviour following ventral hippocampal lesions may stem from altered dorsal hippocampal function warrants consideration and further testing.

In contrast, the link between NGF and hippocampal acetylcholine neurotransmission is not likely to account for the positive relationship between tone-freezing and brain NGF content. The muscarinic cholinergic receptor antagonist, scopolamine, has been shown to disrupt Pavlovian conditioned freezing when administered systemically (Anagnostaras et al., 1995, 1999) or directly into the hippocampus (Wallenstein and Vago, 2001; Gale et al., 2001; Rogers et al., 2004). However, with the exception of one study (Young et al., 1995), this effect of scopolamine is restricted largely to contextual freezing. Here, we did not observe any relationship between context freezing and brain neurotrophin levels.

Conclusion

Although the present correlative approach may be limited in its ability to provide direct information concerning the precise mechanistic processes involved, it has extended the scope of neurotrophin signalling in mental health to the domain of anxiety traits and/or symptoms. Admittedly, anxiety encompasses a wide range of normal as well as pathological behaviour and cognition. The current approach focuses mainly on a sample taken from a normal mouse population, and its relevance to pathological anxiety state awaits further validation. Some of the difficulties in integrating our present findings with existing pharmacological and physiological data may stem from the unique approach of correlative analysis with an emphasis of individual differences as opposed to treatment-induced effects. The latter approach would be necessary for the identification of the mechanisms involved in the correlative links between neurotrophic factors content and anxiety-related behaviour demonstrated here.

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