ONLINE SUPPLEMENT

MAJOR CONTRIBUTION OF THE MEDIAL AMYGDALA TO HYPERTENSION IN BPH/2J GENETICALLY HYPERTENSIVE MICE

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Supplement Materials and Methods

Animals
The genetically hypertensive BPH/2J mice (n=13) and normotensive BPN/3J (n=16) male mice used in the present study came from inbred colonies bred at the Alfred Medical Research and Education Precinct Animal Centre (Generation 15-20) from breeders purchased at generation 20-36 from Jackson laboratories. The original breeding selection program, took place in the 1970’s for at least 23 generations and then brother sister mating followed to create these inbred strains. All mice used in the present study were housed in individual cages with environmental enrichment, in a room with a 12:12 hour light-dark cycle (1am–1pm light/day) with ad libitum access to water and mouse chow (Specialty Feeds, Glen Forrest, Western Australia; 19% protein, 5% fat, 5% fibre, 0.2% sodium). The experiments were approved by the Alfred Medical Research Education Precinct Animal Ethics Committee and conducted in accordance with the Australian Code of Practice for Scientific Use of Animals, in line with international standards.

Radiotelemetry transmitter implantation
Blood pressure (BP) telemetry transmitters (model TA11PA-C10; Data Sciences International, St Paul, MN) were implanted under isoflurane open circuit anesthesia (5% induction and 1.5-2% maintenance) (Forthane, Abbott, Botany, NSW, Australia) delivered via oxygen. Carprofen (5mg/kg)(Rimadyl, Pfizer Australia Pty Ltd, West Ryde, NSW, Australia) was administered subcutaneously just prior to surgery and 24 hours post-surgery for analgesia. A lateral incision and blunt dissection were used to expose the left carotid artery which was temporarily occluded using a non-absorbable silk tie (Dysilk 1-0, Dyneck Pty Ltd, SA, Australia). The catheter of the telemetry device was inserted into the carotid artery and secured using silk ties and the body of the probe was positioned subcutaneously along the right flank. A subcutaneous continuous stitch using an absorbable suture (Polysorb, Covidien, Mansfield, MA) was used to close the incision. Mice were allowed at least ten days recovery prior to BP measurement. Cardiovascular and locomotor recordings were sampled at 1000 Hz using an analog-to-digital data acquisition card (National Instruments 6024E) as described previously.

Lesions of the medial amygdala
Mice were anaesthetized with a combination of 100mg/kg Ketamine (Ketalar, Pfizer), 10mg/kg Xylazine (Ilium Xylazil-20, Smithfield, Australia) and 1.2mg/kg Atropine
Jackson et al. Medial amygdala in BPH/2J hypertensive mice

Mice were placed in the prone position in a stereotaxic apparatus (Angle Two™, Leica, USA) the skull exposed and holes (~1mm) drilled into the skull in the appropriate locations. Four bilateral microinjections (~30nl each) of 10ug ibotenic acid/ 1ul 0.1M NaOH (Tocris bioscience, Bristol, UK) were made into the MeAm via a glass micropipette (~100um tip), connected to a micromanipulator via SV 10 tubing, directed by the computer aided stereotaxic apparatus. Sham controls underwent the same surgery where the micropipette was lowered to the same co-ordinates but no injection performed. Coordinates from bregma: medial/lateral (ML) +/-1.9mm mm, anterior/posterior (AP) -1.7mm dorsal/ventral (DV) -5.4mm; ML +/-2.2mm, AP -1.7mm, DV -5mm; ML +/-2.05mm, AP -1.82mm, DV -5.4mm; ML +/-2.05mm, -1.58mm, DV -5.4mm. Post-operative analgesia was provided by subcutaneous administration of 5mg/kg Carprofen (Pfizer Australia Pty Ltd, West Ryde, NSW, Australia).

**Behavioural stimuli**

Dirty cage-switch stress involves placing mice in a cage previously occupied by another male mouse. This stressor was also conducted for 60 minutes and has been demonstrated to induce sustained pressor responses as well as increased locomotor activity. The dirty cages used for this stress were generally occupied by a male mouse of a different strain for approximately a week beforehand. All behavioural tests are conducted before lesions and following 1 and 3 weeks post lesion. As such the stressors are not considered to be novel when conducted post lesion. Shaker stress involves placing the telemetry receiver and mouse in their home cage onto an orbital shaker (Ratek, Baronia, Victoria, Australia) at a speed of 90 rotations per min for 5 min. Restraint stress involved guiding the mouse into a cylindrical Plexiglas restrainer with a sliding back plate to confine the animal for 60 minutes. Immobilisation minimises the contribution of locomotor activity to cardiovascular response to stress.

**Fos Immunohistochemical Analysis**

Mice were deeply anaesthetized with an intraperitoneal injection of sodium pentobarbitone (100 mg/kg) following 1-hour of dirty cage swap stress, conducted during the light (inactive) period. Animals were perfused transcardially with 20 ml of 0.9 % saline and 60 ml of 4 % paraformaldehyde dissolved in 0.1 M phosphate buffer, pH 7.2 (PB). Subsequently, the brain was removed and postfixed for 1-hour in 20 % sucrose in paraformaldehyde, and placed in 20 % sucrose in PB and refrigerated overnight at approximately 4 °C. Coronal sections (40 µm) were cut on a cryostat and
placed in PB. Free-floating sections were incubated in 10 % normal horse serum at room temperature for 1-hour. Sections were then incubated in primary antibody, sheep anti-c-Fos (Chemicon) diluted 1:2000 in a solution of 2 % normal horse serum and 0.3 % Triton X-100 (Sigma) in PB at room temperature overnight. Sections were washed in PB prior to incubation in biotinylated donkey anti-sheep immunoglobins (1:200, Jackson) in PB containing 2 % normal horse serum for 1-hour. Thereafter, the sections were washed and incubated in avidin-biotin peroxidase complex (1:100, Vector) in PB for 1-hour. Following washes in 0.05 M Tris buffer (pH 7.6), sections were incubated in a solution of 40 mg nickel ammonium sulphate and 50 mg 3-3′ diaminobenzidine hydrochloride per 100 ml Tris buffer for 10 mins, 15 µl of 30 % hydrogen peroxide was added for a further 6-mins. Following final washes, sections were mounted on gelatin coated microscope slides. Bright-field illumination using a Motic BA400 microscope and Motic images plus 2.0 were used to assess the Fos-immunoreactivity in the MeAm as detected by black stained nuclei. Fos staining was counted in two brain sections per coronal atlas plate between -0.94-2.06mm from bregma, within the boundaries of the MeAm as outlined in the mouse brain atlas.

Cardiovascular variability and the cardiac baroreceptor sensitivity
Beat-to-beat data were analysed separately to calculate power spectra using a program written in Labview. The auto- and cross-power spectra were calculated for multiple overlapping (by 50%) segments of MAP and HR using a Fast Fourier transform as adapted for conscious mice. The cardiac baroreflex sensitivity was estimated as the average value of the transfer gain in the frequency band between 0.3 and 0.5 Hz. Baroreflex slope was considered significant if the coherence between MAP and HR across several overlapping segments in the analysed frequency band was >0.4. Data periods with low locomotor activity were chosen (4 from each circadian period) from 48 hour recordings minimizing the influence of physical activity.

Cardiovascular response to ACE inhibition and ganglion blockade
Following a 30 minute baseline, BPH/2J mice (n=3) were administered with an ACE inhibitor, enalaprilat (1.5mg/kg, IP) followed 30 minutes later by the ganglion blocker, pentolinium (5mg/kg, IP). To calculate the effect of enalaprilat, the 30 minute control period immediately preceding the enalaprilat injection was compared with the response 15-30 minutes following enalaprilat injection. To calculate the effect of pentolinium, the 15 minute period immediately preceding the pentolinium injection was used as the
control period and compared with the response 15-30 minutes following the pentolinium injection.

References

Supplementary results

Table S1: Fos counts in the anterior cortical amygdala, basolateral amygdala and central amygdala in BPN/3J and BPH/2J mice following stress in mice with sham or medial amygdala lesions.

<table>
<thead>
<tr>
<th>Brain region</th>
<th>BPN/3J Sham</th>
<th>BPN/3J MeAm lesion</th>
<th>BPH/2J Sham</th>
<th>BPH/2J MeAm lesion</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACo</td>
<td>8.4 ± 0.9</td>
<td>15.8 ± 1.3</td>
<td>10.7 ± 0.9</td>
<td>17.8 ± 0.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BLA</td>
<td>13.3 ± 1.3</td>
<td>15.4 ± 0.6</td>
<td>12.1 ± 1.1</td>
<td>22.3 ± 0.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CeAm</td>
<td>4.7 ± 0.8</td>
<td>9.3 ± 0.6</td>
<td>4.7 ± 0.4</td>
<td>10.3 ± 0.7</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Anterior cortical nucleus of the amygdala (ACo), basolateral amygdala (BLA), central amygdala (CeAm). P value represents a within strain comparison of counts in mice following medial amygdala (MeAm) lesions compared with sham.
Figure S1: Line graphs show hourly averages of mean arterial pressure (MAP), heart rate (HR, beats per minute) and locomotor activity (Act) during the dark (active, outer panels) and light (inactive, middle panel) phases in BPN/3J (Left, grey n=7) and BPH/2J (Right, black, n=7). Line graphs include measurements at baseline (pretreatment, open circles) and following A; MeAm lesion surgery and B; sham surgery (average of 1&3 weeks post lesion, closed circles). Histograms are mean change ± SEM from baseline post lesion during the light (inactive) period (unfilled) and dark (active) period (hatched) in BPN/3J (grey) and BPH/2J (black). Difference from baseline *P<0.05; **P<0.01; ***P<0.001.
Figure S2: Mean arterial pressure (MAP), heart rate (HR) and locomotor activity response to administration of enalaprilat in BPH/2J (black) mice during the light inactive period (left, n=3) and dark active period (right, n=3). Each point represents the mean value averaged across a 5-minute period. The dashed vertical reference line represents the time-point of administration of treatment. Shaded area represents the period analyzed for comparison of the effect of treatment. Bar graphs represent average changes in MAP in response to agents in BPH/2J mice at baseline (B) and after MeAm lesions (L). Bar graphs values are mean±SEM. Significance refers to effect of lesion *P<0.05.
Figure S3: Mean arterial pressure (MAP), heart rate (HR) and locomotor activity response to administration of pentolinium following pre-treatment with enalaprilat in BPH/2J (black) mice during the light inactive period (left, n=3) and dark active period (right, n=3). Each point represents the mean value averaged across a 5-minute period. The dashed vertical reference line represents the time-point of administration of treatment. Shaded area represents the period analyzed for comparison of the effect of treatment. Bar graphs represent average changes in MAP in response to agents in BPH/2J mice at baseline (B) and after MeAm lesions (L). Bar graphs values are mean±SEM. Significance refers to effect of lesion *P<0.05, **P<0.01.
**Figure S4:** Line graph represent average mean arterial pressure (MAP) and heart rate (HR, beats per minute) responses before and during restraint stress at baseline in BPN/3J (grey) and BPH/2J mice (black) (Baselines pooled from sham and lesion groups). Each dot represents mean ± SEM averaged across 10-minute periods. Bar graphs represent average MAP and HR response to the stimuli at baseline (B) and average response following sham surgery (S) or MeAm lesion (L). Post sham and lesion values are an average of measurements at 1 and 3 weeks post-surgery. Values are mean ± SEM. *, $P<0.05$ represents comparison of post-treatment responses with baseline responses in each strain.
Figure S5: Line graph represent average mean arterial pressure (MAP), heart rate (HR, beats per minute) and locomotor activity (Act) responses before and during shaker stress in BPN/3J (grey) and BPH/2J mice (black) at baseline (Baselines pooled from sham and lesion groups). Each dot represents mean ± SEM, averaged across a 30-second period. Bar graphs represent average MAP, HR and locomotor activity response to the stimuli at baseline (B) and average response following sham surgery (S) or MeAm lesion (L). Post sham and lesion values are an average of measurements at 1 and 3 weeks post-surgery. Values are mean ± SEM.