Regional distribution of *Ginkgo biloba*-induced c-Fos immunoreactivity

P.E. Mallet\textsuperscript{a,*}, C.A. Moore\textsuperscript{b}, M.T. Collie\textsuperscript{b}, E. Satvat\textsuperscript{a}

\textsuperscript{a}Department of Psychology, Wilfrid Laurier University, Waterloo, Ontario, Canada N2L 3C5  
\textsuperscript{b}School of Psychology, University of New England, Armidale, NSW 2351, Australia

Abstract

A growing literature supports the notion that *Ginkgo biloba* has cognitive enhancing and anxiolytic properties; however, its effects on neuronal populations have yet to be characterized. The present study used c-Fos immunoreactivity (Fos-IR) to characterize functional activity in selected brain regions following administration of a standardized *Ginkgo biloba* extract. Because *Ginkgo* is typically consumed orally, Exp 1 sought to identify patterns of neural activity induced by oral administration. To ensure that the alterations in functional neural activity observed in Exp 1 were not simply due to novel gustatory experience, Exp 2 characterized patterns of Fos-IR following intraperitoneal administration of *Ginkgo*. Rats were habituated to handling and experimental conditions. In Exp 1, rats self-administered 150 mg/kg *Ginkgo* or vehicle alone (strawberry jam) orally. In Exp 2, rats were injected with *Ginkgo* (2.5 or 25 mg/kg, i.p.) or vehicle (0.3% gum Arabic). Animals were anaesthetized and perfused transcardially. Brains were sectioned, immunostained using a c-Fos antibody, then the number of labelled cells was quantified microscopically in selected brain regions. In both experiments *Ginkgo* increased Fos-IR in numerous brain regions including the insular cortex and amygdala. Intraperitoneal administration induced Fos-IR in some additional regions including the nucleus accumbens and dentate gyrus. Results provide important preliminary data serving to identify several candidate neural sites involved in the cognitive enhancing and anxiolytic effects of *Ginkgo biloba*.

Keywords: *Ginkgo biloba*; Immunohistochemistry; c-Fos

Introduction

EGb 761 is a water–acetone extract of dried *Ginkgo biloba* leaves, standardized to contain 24% flavonoid glycosides, 6% terpenoids, and less than 9% proanthocyanidins and organic acids (DeFeudis and Drieu, 2000; Maclennan et al., 2002). Extracts of *Ginkgo biloba*, such as EGB 761, are commonly used to increase blood circulation peripherally and centrally, and to protect the lipid portion of cellular membranes against damage induced by free radicals (reviewed by Ahlemeyer and Kriegstein, 2003). A growing literature supports the notion that *Ginkgo biloba* has cognitive enhancing properties. For example, studies using rats have shown that *Ginkgo biloba* facilitates olfactory recognition (Wirth et al., 2000), enhances performance in a radial maze (Winter, 1998), and facilitates learning in a water maze (Wang et al., 2006). In humans, extracts of *Ginkgo biloba* have been found to improve cognitive function in young healthy individuals (Stough et al., 2001), in the elderly (Winther et al., 1998), and in patients with Alzheimer’s disease (Maurer et al., 1997; Oken et al., 1998). Studies also generally support the notion that extracts of *Ginkgo biloba* reduce basal...
(Kuribara et al., 2003) or stress-induced anxiety (Ward et al., 2002), and attenuate the cognitive impairments induced by exposure to stress (Walesiuk and Braszko, 2007; Walesiuk et al., 2005, 2006).

The neural mechanisms responsible for the cognitive and behavioural effects of *Ginkgo biloba* extracts are poorly understood; however, several candidate modes of action have so far been identified. Administration of *Ginkgo biloba* dose-dependently inhibits acetylcholinesterase in vitro (Das et al., 2002), and increases cortical blood flow in vivo (Kriegstein et al., 1986), although subcortical blood flow influences and regional specificity have not yet been studied. In aged rats, oral administration of an extract of *Ginkgo biloba* increases the density of hippocampal muscarinic receptors (Taylor, 1986). Other neurotransmitter systems may also be affected by exposure to *Ginkgo biloba* including adrenergic, dopaminergic, serotonergic, glutamatergic, and GABAergic systems (reviewed by Ahlemeyer and Kriegstein, 2003). Hippocampal electrophysiological alterations have also been noted. For example, EGB 761 was found to reduce the population spike threshold and increase the early phase of long-term potentiation in aged mouse hippocampal slices (Williams et al., 2004). Furthermore, administration of EGB 761 to aged rats increased the magnitude of inducible long-term potentiation in the CA1 region (Wang et al., 2006).

The aim of this study was to further characterize the neural effects of an extract of *Ginkgo biloba* by using c-Fos immunoreactivity (Fos-IR) in rats to quantify functional activity in several brain regions known to be involved in learning and memory. Expression of the immediate early gene *c-fos* is largely dependent on synaptic stimulation (reviewed by Kaczmarek and Chaudhuri, 1997). The immunohistochemical localization of c-Fos can therefore serve as a useful neurobiological tool to map functional activity. Because *Ginkgo biloba* is typically consumed orally, the first experiment sought to identify patterns of neural activity induced by orally self-administered *Ginkgo*. To control the influence and novel gustatory experience on *c-fos* expression following oral administration, a second experiment served to characterize patterns of Fos-IR following parenteral *Ginkgo* administration.

**Materials and methods**

Animals were treated in accordance with the National Health and Medical Research Council’s “Australian code of practice for the care and use of animals for scientific purposes” (7th edition, 2004) and the “Principles of laboratory animal care” (National Research Council, 1996). Approval for this research was obtained from the University of New England Animal Ethics Committee.

**Subjects**

Forty male albino Wistar rats (16 for Exp 1 and 24 for Exp 2, mean weight 320 g), bred at the University of New England, were group-housed in a temperature-controlled room (21 °C) maintained on a 12:12 h reversed light:dark cycle (lights on at 19:00 h). Experiments were conducted during the dark cycle. Animals had free access to lab chow (Barastoc, Ridley AgriProducts, Australia) and tap water throughout the study. Rats were handled for 7 days prior to the commencement of the experiment.

**EGB 761 preparation and administration**

An injectable solution was prepared by mixing the *Ginkgo biloba* extract EGB 761 (Ginkgoselect, Indena S.p.A, Milan, Italy) in 0.3% Gum Arabic (Sigma-Aldrich, Castle Hill, NSW). The solution was stored at 4 °C until needed. Ginkgoselect is composed of EGB 761 – standardized to contain 24% ginkgoflavonglucosides, 6% ginkgolides and bilobalide, and less than 5 ppm ginkgoic acids – complexed with soy phospholipids at a ratio of 1:2 (w/w) to increase bioavailability. Injections were administered intraperitoneally in a volume of 2 ml/kg body weight at doses of 2.5 and 25 mg/kg body weight. Doses are expressed to reflect the EGB 761 content of the phospholipid complex. The i.p. administration of a similar dose of Ginkgoselect was found to enhance acquisition of a double Y-maze task (Satvat and Mallet, under review).

A *Ginkgo biloba* suspension was also prepared for oral consumption using commercial water-soluble EGB 761 tablets (AIM Ginkgo 3000™, AIM International, Somerton Victoria, Australia). Tablets were crushed into a fine powder and mixed with strawberry jam (Savings brand, Coles Myer Ltd., Tooronga, Victoria, Australia) such that each gram of jam contained 30 mg EGB 761. Rats were fed 5 g jam per kg body weight per dose, yielding an EGB 761 dose of 150 mg/kg.

**Procedure**

**Experiment 1: oral administration**

To reduce flavour neophobia, a glass dish containing 30 g strawberry jam without EGB 761 was placed in each group home cage containing six rats on each of 2 consecutive days.

The next phase of the experiment, lasting 21 days, served to reduce *c-fos* expression in response to novelty, handling, and experimental procedures. Each day animals were weighed and placed individually in 40 × 30 × 25 cm seclusion cages for 3 h, in a dimly lit (two 40 W incandescent red lamps) quiet room with soft pink noise (noise generator model K2135, Altronics,
Perth, WA, Australia). Seclusion cages contained a dish with 5 g/kg strawberry jam, which later served as the vehicle for EGB 761. Placement in the seclusion cages was staggered to habituate the animals to the experimenter entering the room at 10 min intervals, which was required for the precise timing of the perfusions later in the experiment.

On the next day the experiment was conducted as usual, except that the strawberry jam was replaced with the EGB 761 jam mixture for half the rats \( n = 8 \). At the end of the 3-h seclusion session, rats were deeply anesthetized with sodium pentobarbital (120 mg/kg, i.p.), and then perfused transcardially first with 100 ml phosphate-buffered saline (0.9%, pH 7.2), then with 150 ml phosphate-buffered paraformaldehyde (4%, pH 7.2). To ensure group differences in \( c-fos \) expression were not related to circadian rhythms, odd-numbered rats in the sequence were assigned to the vehicle group, and even-numbered rats in the sequence were assigned to the EGB 761 group.

**Experiment 2: intraperitoneal administration**

On each of 4 consecutive days, animals were weighed, injected with 0.3% gum Arabic (i.p.), then placed individually in \( 40 \times 30 \times 25 \) cm seclusion cages in a dimly lit quiet room (details above) for 2 h. On the following day, the experiment was conducted as usual, except that rats were injected with the gum Arabic vehicle \( n = 8 \), 2.5 mg/kg EGB 761 \( n = 8 \), or 25 mg/kg EGB 761 \( n = 8 \) prior to the 2-h seclusion session. Rats were then anesthetized and perfused as described above. To enforce that group differences in Fos-IR were not related to circadian rhythms, the order of treatments was counterbalanced such that one rat from each group was perfused every 45 min (i.e., one perfusion was performed every 15 min).

**Immunohistochemistry**

Immunohistochemical staining was performed in a manner similar to that described earlier (Singh et al., 2005, 2006). Following perfusions, brains were extracted using brightfield microscopy at 200 \( \times \) magnification. The atlas of Paxinos and Watson (1997) was used to identify the brain regions shown in Fig. 1. A \( 10 \times 10 \) square graticule was positioned over a standardized region of each structure and the number of labelled nuclei within the graticule, which covered a 500 \( \times \) 500 \( \mu \)m area, was counted manually. Only round and oval nuclei that were completely black were counted. Structures examined included the caudate-putamen (CPu): central, dorsal, dorsomedial, and ventrolateral regions; cortex: cingulate, insular, and piriform regions; nucleus accumbens (nAcc): core and shell regions; lateral septal nucleus, ventral part (LSV); bed nucleus of the stria terminalis, lateral division, dorsal region (BNST); thalamus: paraventricular nucleus (PV) and dorsomedial; amygdala: basolateral nucleus (BNA), central nucleus (CEA), and medial nucleus (MNA); hippocampal formation: dentate gyrus, CA1 and CA3 regions; and the premammillary nucleus.

**Microscopy**

Each microscope slide was coded to ensure “blind” counting. A total of 20 brain regions were examined using brightfield microscopy at 200 \( \times \) magnification. The atlas of Paxinos and Watson (1997) was used to identify the brain regions shown in Fig. 1. A \( 10 \times 10 \) square graticule was positioned over a standardized region of each structure and the number of labelled nuclei within the graticule, which covered a 500 \( \times \) 500 \( \mu \)m area, was counted manually. Only round and oval nuclei that were completely black were counted. Structures examined included the caudate-putamen (CPu): central, dorsal, dorsomedial, and ventrolateral regions; cortex: cingulate, insular, and piriform regions; nucleus accumbens (nAcc): core and shell regions; lateral septal nucleus, ventral part (LSV); bed nucleus of the stria terminalis, lateral division, dorsal region (BNST); thalamus: paraventricular nucleus (PV) and dorsomedial; amygdala: basolateral nucleus (BNA), central nucleus (CEA), and medial nucleus (MNA); hippocampal formation: dentate gyrus, CA1 and CA3 regions; and the premammillary nucleus.

**Statistical analysis**

In Exp 1 (oral administration), the number of Fos-immunoreactive cells was compared between groups using unpaired \( t \)-tests. In Exp 2 (i.p. administration), the number of labelled cells was compared between groups using one-way analyses of variance (ANOVA). When a significant ANOVA was found, groups were further compared using post hoc Tukey tests. Because the homogeneity of variance and normality assumptions were frequently violated, independent groups randomization tests were also conducted using the program NPFact (May et al., 1993) to confirm the outcome of the parametric tests.
Results

The numbers of Fos-immunoreactive cells for each brain region examined are shown in Table 1. In addition, representative photomicrographs of Fos-immunoreactive nuclei are presented in Fig. 2. In all cases but one, the randomization tests yielded the same outcome as the parametric tests, so for ease of interpretation only the outcome of the randomization tests are shown. In one case the ANOVA was marginally significant, but the randomization test was not. Because the ANOVA homogeneity of variance assumption was violated, the non-significant outcome of the randomization test was adopted.

Oral administration of EGb 761 (Exp 1) increased Fos-IR moderately, but significantly, in the insular cortex \((t_{13} = 2.67, p < 0.05)\) and amygdala (basolateral and central nuclei; \(t_{14} = 5.56, p < 0.001\) and \(t_{14} = 5.56, p < 0.05\), respectively). The i.p. administration of EGb 761 (Exp 2) also significantly increased Fos-IR in several brain regions including the prelimbic \((F_{2,21} = 8.18, p < 0.01)\) and insular cortices \((F_{2,21} = 4.38, p < 0.05)\), nAcc shell \((F_{2,21} = 4.01, p < 0.05)\), LSV \((F_{2,21} = 4.71, p < 0.05)\), BNST \((F_{2,21} = 5.29, p < 0.05)\), PV \((F_{2,21} = 7.62, p < 0.01)\), CEA \((F_{2,21} = 7.09, p < 0.01)\), and dentate gyrus \((F_{2,21} = 2.62, p < 0.05)\). Outcomes of the post hoc pairwise comparisons are presented in Table 1.

Fig. 1. Schematic diagrams of coronal sections of the rat brain (adapted from Paxinos and Watson, 1997). The number of Fos-immunoreactive nuclei was quantified within the areas numbered and shaded in grey (shown to scale). Labels correspond to the brain regions listed in Table 1.
Table 1. Mean (±S.E.) number of Fos-immunoreactive cells per mm²

<table>
<thead>
<tr>
<th>Region</th>
<th>Bregma</th>
<th>Exp 1: oral</th>
<th>Exp 2: intraperitoneal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Vehicle 150 mg/kg</td>
<td>Vehicle 2.5 mg/kg</td>
</tr>
<tr>
<td>1. Cortex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) Prelimbic</td>
<td>+3.20</td>
<td>43.2±16.0</td>
<td>71.4±17.0</td>
</tr>
<tr>
<td>(b) Infrafimbic</td>
<td>+3.20</td>
<td>52.8±18.0</td>
<td>86.3±25.1</td>
</tr>
<tr>
<td>(c) Cingulate</td>
<td>+1.00</td>
<td>26.9±6.2</td>
<td>31.5±6.6</td>
</tr>
<tr>
<td>(d) Insular</td>
<td>+1.00</td>
<td>29.7±4.4</td>
<td>103.0±25.3*</td>
</tr>
<tr>
<td>(e) Piriform</td>
<td>+1.00</td>
<td>27.4±4.6</td>
<td>33.5±4.1</td>
</tr>
<tr>
<td>2. Caudate-putamen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) Central region</td>
<td>+1.00</td>
<td>1.1±0.7</td>
<td>7.0±4.4</td>
</tr>
<tr>
<td>(b) Dorsal region</td>
<td>+1.00</td>
<td>2.3±1.5</td>
<td>2.5±1.1</td>
</tr>
<tr>
<td>(c) Dorsomedial region</td>
<td>+1.00</td>
<td>10.3±3.1</td>
<td>13.5±1.7</td>
</tr>
<tr>
<td>(d) Ventrolateral region</td>
<td>+1.00</td>
<td>6.3±4.4</td>
<td>11.5±3.6</td>
</tr>
<tr>
<td>3. Nucleus accumbens</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) Core region</td>
<td>+1.00</td>
<td>8.0±3.6</td>
<td>26.0±6.9</td>
</tr>
<tr>
<td>(b) Shell region</td>
<td>+1.00</td>
<td>19.4±2.7</td>
<td>25.5±6.1</td>
</tr>
<tr>
<td>4. Lateral septal nucleus, ventral part</td>
<td>+1.00</td>
<td>46.9±19.5</td>
<td>53.0±11.5</td>
</tr>
<tr>
<td>5. BNST lateral division, dorsal</td>
<td>−0.26</td>
<td>33.7±10.0</td>
<td>53.3±12.6</td>
</tr>
<tr>
<td>6. Thalamus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) Paraventricular nucleus</td>
<td>−2.30</td>
<td>121.7±19.0</td>
<td>167.5±16.9</td>
</tr>
<tr>
<td>(b) Dorsomedial</td>
<td>−2.30</td>
<td>14.9±7.8</td>
<td>23.5±5.6</td>
</tr>
<tr>
<td>7. Amygdala</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) Basolateral nucleus</td>
<td>−2.30</td>
<td>15.5±4.0</td>
<td>47.5±4.2**</td>
</tr>
<tr>
<td>(b) Central nucleus</td>
<td>−2.30</td>
<td>44.0±14.8</td>
<td>100.0±15.4*</td>
</tr>
<tr>
<td>(c) Medial nucleus</td>
<td>−2.30</td>
<td>23.0±5.2</td>
<td>36.0±7.4</td>
</tr>
<tr>
<td>8. Hippocampal formation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) Dentate gyrus</td>
<td>−3.30</td>
<td>21.5±5.5</td>
<td>36.5±5.6</td>
</tr>
<tr>
<td>(b) CA1</td>
<td>−3.60</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>(c) CA3</td>
<td>−3.60</td>
<td>6.5±2.0</td>
<td>9.1±2.1</td>
</tr>
<tr>
<td>9. Pre-mammillary nucleus, ventral</td>
<td>−3.80</td>
<td>7.0±3.8</td>
<td>16.0±4.6</td>
</tr>
</tbody>
</table>

*p<0.05, **p<0.01 vs. vehicle; †p<0.05, ‡‡p<0.01 vs. 2.5 mg/kg.

Discussion

Results revealed that both oral and parenteral administration of EGB 761 increased c-fos expression in selected limbic and related structures, suggesting that EGB 761 increases neural activity in a region-specific manner. EGB 761 significantly increased Fos-IR in the insular cortex and CEA using both routes of administration. However, for some brain regions the pattern of neural activation differed depending on the route of administration. For example, Fos-IR was significantly increased in the PV, nAcc shell, and dentate gyrus following i.p. administration, but not following oral administration of EGB 761. Conversely, Fos-IR was increased significantly in the BNA by oral, but not i.p. administration of EGB 761. This should not be taken as evidence that different routes of administration produce unique neural (and consequently behavioural) effects. Indeed a direct comparison of these two routes of administration is not possible given differing pharmacodynamics and the necessary procedural differences required to vary the route of administration (e.g., dose, injection, exposure to jam). Rather, Exp 1 served to characterize neural activity induced by oral administration given that this is the most commonly employed route of administration, while Exp 2 served to demonstrate that many of the observed changes in Fos-IR found in Exp 1 cannot be simply explained by novel gustatory experience (i.e., a noticeable change in jam flavour by the addition of EGB 761).

Growing evidence suggests a critical role for the CEA and insular cortex in learning and memory. For example, the insular cortex has been shown to be involved in incentive memory (Balleine and Dickinson, 2000), the consolidation of object memory (Bermudez-Rattoni et al., 2005), the acquisition and consolidation...
of inhibitory avoidance (Mello e Souza et al., 2001; Miranda and McGaugh, 2004), and the consolidation of conditioned taste aversion (Miranda and McGaugh, 2004). The CEA plays a critical role in the acquisition, consolidation, and expression of Pavlovian fear conditioning (Wilensky et al., 2006). Thus, the finding that acute administration of EGB 761 increased neural activity in the insular cortex, CEA, and dentate gyrus indicates that limbic structures may have potentially important roles in the long-term neurocognitive effects of EGB 761.

The significant increase in Fos-IR found in the dentate gyrus is particularly interesting given the important role of the hippocampal formation in learning and memory (Squire et al., 2004). It is not, however, clear why the increase in Fos-IR was specific to the i.p. route of administration, but this may have been related to the different time courses and doses of EGB 761 used in the two experiments. That is, the time between administration of EGB 761 and perfusion was 1 h longer in the experiment using oral administration to allow for the slower rate of absorption using this route of administration. It is worth noting that although the regional activity in the dentate gyrus was not significantly increased by oral administration of EGB 761, a non-significant trend ($p = 0.088$) was observed. Furthermore, different doses were used in each experiment. In Exp 1, the dose used was 150 mg/kg because pilot studies revealed that this dose approached the maximum concentration in the jam vehicle tolerated by rats. That is, higher concentrations were not well consumed, presumably because of an undesirable taste. In Exp 2, doses were 2.5 and 25 mg/kg. The higher dose approached the maximum quantity of EGB 761 that could be suspended in vehicle using an injection volume of 2 ml/kg. Further studies comparing plasma and brain concentrations of EGB 761 constituents following both routes of administration and time courses would be useful to clarify this point.

The increase in Fos-IR in the PV and prelimbic cortex are noteworthy given the role of midline intralaminar nuclei in attention. Thus, PV efferents project to the amygdala, nucleus accumbens, and ventral aspects of the medial prefrontal cortex (reviewed by Huang et al., 2006), all of which are limbic regions involved in motivation and attention. Fos-IR in the PV and prelimbic cortex was significantly increased by i.p. EGB 761, but the increase following oral administration fell short of statistical significance in both cases. The diminished drug effect following oral administration despite a longer habituation period may be dose, route, or time course related, but is more likely due to the high baseline level of Fos-IR observed in the vehicle group (oral consumption of jam), which may have been a consequence of consuming a highly palatable food. Support for this notion comes from the finding that PV c-fos expression is increased by anticipatory feeding (Nakahara et al., 2004). It is therefore possible that at least some of the cognitive enhancing effects of EGB 761 may be attributed to enhanced cortical arousal and attention.

It is not possible to determine conclusively from the present results alone whether the increase in Fos-IR

Fig. 2. Photomicrographs showing Fos-immunoreactive cells in the central nucleus of the amygdala from representative animals treated with vehicle, 2.5 mg/kg or 25 mg/kg EGB 761 from Exp 2. Scale bar = 100 µm.
found in the BNA following oral consumption of EGB 761 was the result of a central pharmacological effect of EGB 761, or was simply caused by the subjective experience of the novel taste of EGB 761. However, the finding that Fos-IR was not increased significantly in this brain region by parenteral administration of EGB 761 suggests that taste novelty cannot be discounted. In support of this notion, it has been shown that amygdalar c-fos expression is increased by exposure to a novel saccharin solution in mice (Montag-Sallaz et al., 1999) and rats (Koh et al., 2003).

The nature of the neuroplastic changes responsible for the cognitive enhancing effects of chronic administration of EGB 761 have yet to be characterized. Nonetheless, results from the present study provide novel evidence that acute administration of EGB 761 induces neural activity in a region-specific manner. Given that the beneficial cognitive effects of EGB 761 are typically more pronounced following chronic dosing (reviewed by Müller and Chatterjee, 2003), it remains the goal of future research to determine whether chronic alterations in regional activity by EGB 761 induces longer-term neural plasticity responsible for cognitive improvements.

Acknowledgements

This study was supported by the Natural Sciences and Engineering Research Council of Canada, and a grant from the University of New England.

References


