Mesocortical dopamine and HPA axis regulation: Role of laterality and early environment

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ABSTRACT
The infralimbic (IL) cortex is importantly involved in regulating behavioral and physiological responses to stress, including those of the hypothalamic–pituitary–adrenal (HPA) axis. The mesocortical dopamine (DA) system is an important afferent modulator of this region, is highly stress sensitive and frequently shows functional hemispheric asymmetry. Postnatal handling stimulation facilitates development of cortical asymmetry and is also associated with optimal HPA axis regulation. The present study examines the poorly understood role of the mesocortical DA system in regulating HPA axis function in adult rats which were handled (H) or nonhandled (NH) postnatally. In the first experiment, unilateral intra-IL cortex injection of the DA (D1/D2) antagonist α-flupenthixol into either hemisphere significantly exaggerated the restraint stress-induced increases in plasma adrenocorticotrophic hormone and corticosterone in NH rats. In H rats, the same effect was lateralized to the right IL cortex. In a second experiment, post mortem neurochemical analysis of DAergic measures in the IL cortex was conducted in H and NH animals following either acute or repeated (5 times) restraint stress. DAergic measures in the right IL cortex were significantly correlated with reduced stress hormone activation in both H and NH rats, especially in repeatedly restrained rats. However, while H rats showed a significant rightward shift in DA metabolism with repeated stress experience, NH rats shifted DA metabolism to the left. It is suggested that, during stress, mesocortical DA release normally acts in an adaptive, negative feedback capacity preventing excessive HPA activation and, with repeated stress, the right IL cortex is particularly important in this capacity. As well, the selective enhancement of DA metabolism in the right IL cortex of H rats may underlie, in part, their typically superior ability to adapt to stress and constrain HPA activity.

1. Introduction
The hypothalamic–pituitary–adrenal (HPA) axis is of fundamental importance in responding and adapting to stressful challenges. However, chronic activation of this system or exposure to abnormally elevated levels of circulating glucocorticoids is maladaptive at many levels. Stress-related psychiatric conditions like major depression are associated with excessive HPA activity and/or deficient feedback regulation (Barden et al., 1995; Plotsky et al., 1998; Wong et al., 2000). Such conditions have also been frequently associated with functional abnormalities in the prefrontal cortex or PFC.
(Davidson, 1998; Drevets et al., 1997; Martinot et al., 1990; Schaffer et al., 1983; Sullivan and Gratton, 2002b). While it is recognized that the PFC plays a complex role in the regulation of stress and emotional states, its relationship with stress-related neuroendocrine function is not well understood.

Rodent studies have shown that the medial PFC is one of the key brain regions involved in negative feedback regulation of the HPA axis (Diòrio et al., 1993). Lesioning the PFC in rats has been reported to enhance, suppress or not affect stress-induced HPA activity (Brake et al., 2000; Crane et al., 2003; Diòrio et al., 1993; Figueiredo et al., 2003; Sullivan and Gratton, 1999). In general, lesions of more dorsomedial (prelimbic and anterior cingulate) regions appear to suppress HPA activity, while those in more ventromedial or infralimbic (IL) cortex appear to facilitate HPA activity (reviewed in Sullivan, 2004). As well, the role of the medial PFC in modulating HPA function is stressor-specific and particularly important in “processive” stressors involving higher brain integration as opposed to more “systemic” stressors (Crane et al., 2003; Diòrio et al., 1993; Figueiredo et al., 2003; Herman et al., 1996).

The ventromedial or IL region of the PFC appears to facilitate not only HPA activation, but also autonomic and behavioral arousal in times of stress (Frysztak and Neafsey, 1991, 1994; Lacroix et al., 2000; Shah and Treit, 2003; Sullivan and Gratton, 1999, 2002a). This region has many direct anatomical links with subcortical limbic and brainstem sites regulating physiological stress responses and emotional expression (Hurley et al., 1991; Sesack et al., 1989; Takagishi and Chiba, 1991; Terreberry and Neafsey, 1987). The mesocortical dopamine (DA) system originating in the ventral tegmental area sends a particularly dense innervation to the IL cortex (Van Eden et al., 1987). While the mesocortical DA system is now known to project to diverse cortical regions, we use the term here to refer to the ventromedial PFC projection, which is potently activated by even mild stress (Abercrombie et al., 1989; Deutch and Roth, 1990; Feenstra et al., 1995; Sullivan, 2004; Sullivan and Szeczenman, 1995; Sullivan et al., 1998; Thiel and Schwarting, 2001), we wished to examine the extent to which mesocortical DA modulation of HPA activation is lateralized as a function of early H stimulation. To this end, we studied the effects of DAergic blockade separately in the left and right IL cortex in both H and NH rats, with the hypothesis that any effects of DAergic blockade would show more right lateralization in H rats.

Exp. 2 of the present study examines the relationship between mesocortical DA function and HPA activation following repeated stress exposure in H and NH rats. HPA activation normally habituates after repeated exposure to the same stress (Bhatnagar and Meaney, 1995; Bhatnagar et al., 2005; Lachuer et al., 1994, and unpublished observations), particularly so in H rats. As such, we wished to examine changes in postmortem measures of DA function in the left and right IL cortex following repeated vs. acute restraint, with the hypothesis that H rats would again show more functional lateralization and possibly an upregulation of right mesocortical DA activity with stressful experience.

2. Results

2.1. Experiment 1

All rats included in Exp. 1 had injection sites within the boundaries of the IL cortex as defined by the atlas of Paxinos and Watson (1988, see Fig. 1). Within sham groups, no neuroendocrine measures differed as a function of left- vs. right-sided vehicle injection, so these “subgroups” are combined as a single sham group within each treatment condition.

For both ACTH and CORT, the expected large sample effects were observed across all groups, confirming the usual effectiveness of the restraint stressor in elevating hormone levels relative to pre-stress levels (in both Exp. 1 and 2). For ACTH, both a significant Group Effect ($F_{2,56} = 5.98$, $P = 0.004$) and Group × Treatment interaction ($F_{2,56} = 3.24$, $P = 0.047$) were observed (Fig. 2). In NH rats, both right and left DAergic blockade significantly increased ACTH levels at all time points ($P < 0.05$), except at 80 min where right injections just missed significance ($P < 0.10$). Conversely, in H rats, only right-sided injections were effective in increasing ACTH levels, significantly so at the peak stress (20 min) sample ($P < 0.01$).

For plasma CORT (Fig. 3), the only significant effect was a Group × Treatment × Sample interaction ($F_{4,110} = 4.14$, $P < 0.01$).
P = 0.020). CORT levels differed only at the stress recovery time point such that, in NH rats, both right (P < 0.05) and left (P < 0.01) injections of α-flupenthixol increased plasma CORT levels relative to shams. Left and right injections did not differ from each other (P > 0.05). In H rats, however, right side DA blockade elevated CORT levels relative to both shams (P < 0.01) and left side blockade (P < 0.05), while shams and left-sided DA blockade did not differ from each other.

2.2. Experiment 2

Exp. 2 revealed that H and NH rats respond differently to repeated stressful experience, both in terms of neuroendocrine activation and mesocortical DA function. In terms of ACTH activation (Fig. 4), a significant Treatment × Stress interaction was observed (F_{1,29} = 10.13, P = 0.003), such that while H rats tended nonsignificantly to habituate to the peak stress response, ACTH levels in NH rats significantly sensitized at all three sample points. At both the pre-stress and recovery time points, NH-RR rats were significantly higher than NH-AR rats and both H groups (P < 0.05). At the peak sample, NH-RR were elevated relative to NH-AR and H-RR (P < 0.05).

Stress-induced CORT levels showed a somewhat similar pattern, although the Treatment × Stress interaction failed to reach statistical significance (P = 0.070). There was however a significant Treatment × Sample effect (F_{2,58} = 6.11, P = 0.004) and a Stress × Sample effect (F_{2,58} = 5.73, P = 0.005). At the pre-stress time point, NH-RR rats had significantly higher CORT levels than NH-AR and H-AR groups (P < 0.05). At the peak stress sample, NH-RR rats differed from NH-AR rats (P < 0.05) (Fig. 5).

Neurochemical indices of DAergic function in the IL cortex diverged as a function of repeated stress and side (Fig. 6).

Fig. 2 – Effects of intracortical α-flupenthixol on plasma ACTH levels in NH (above) and H (below) rats. In NH rats, unilateral DA receptor blockade in either the left or right IL cortex was equally effective in increasing ACTH levels across the sampling period. In contrast, only right-sided blockade was effective in elevating the peak stress levels of ACTH in H rats (see text for details). Shams received an equal volume of artificial CSF vehicle in the left or right IL cortex. The black bar denotes the 20 min restraint stress (P < 0.05, n = 10–12 per group).
Overall, tissue content of DA was greater in H than NH rats (Treatment effect, F₁,29 = 6.60, P = 0.016). While NH rats showed no hemispheric asymmetry in either stress condition, H rats showed a strong tendency to shift from a left-sided bias following acute stress to a right-sided bias following repeated stress, although this Treatment × Stress × Side interaction failed to reach statistical significance (P = 0.09). However, the same interaction was highly significant for the levels of DOPAC (F₁,29 = 10.0, P = 0.004). While H rats showed a striking shift in metabolite levels from an initial leftward bias to a rightward bias following repeated stress, NH rats actually increased metabolism in the left IL cortex with repeated stressful experience. Group comparisons revealed that DOPAC levels in the left IL cortex were significantly greater in NH-RR and H-AR rats compared to NH-AR and H-RR groups (P < 0.05). In the right IL cortex, DOPAC levels were higher in H-RR rats than all other groups (P < 0.05). Within group side comparisons (with paired t tests) showed an L > R asymmetry in NH-RR animals, but an R > L asymmetry in H-RR rats (P < 0.05). Tissue levels of HVA showed a similar pattern to DOPAC (data not shown), but like DA this 3-way interaction failed to reach significance (P = 0.11). As well, neither the levels of NE nor 5-HIAA showed any significant effects or interactions, and, although each tended to demonstrate the same rightward shift in the H-RR rats, this was less pronounced than with the DAergic measures.

Finally, correlational analyses within individual groups revealed numerous significant correlations between DAergic measures and ACTH or CORT levels at the various sample points, suggesting a close functional relationship between mesocortical DA activity and neuroendocrine regulation (see Table 1). The pattern of these relationships, however, varied somewhat across the different groups. In general, DAergic measures were more consistently related to stress hormone levels in groups repeatedly restrained than in the acute restraint groups. As well, DAergic measures in the right IL cortex were predominantly correlated with reduced stress hormone levels, while left-sided measures were more often positively correlated with ACTH and CORT levels.

Fig. 3 - Effects of intracortical α-flupenthixol on plasma CORT levels in NH (above) and H (below) rats. The effects of DAergic blockade on CORT levels were restricted to the stress recovery time point. In NH rats, both left and right blockade kept plasma CORT significantly elevated relative to Shams (artificial CSF vehicle), while in H rats only right blockade was effective. See text for statistical details (*P < 0.05, n = 10–12 per group).

Fig. 4 - Effects of repeated (5) restraint stress on plasma ACTH in NH (above) and H (below) rats. In relation to a single, acute restraint, H rats tended to habituate to the peak stress levels of ACTH, while NH rats showed a sensitization of stress hormone levels across all time points following repeated stress exposure (see text for statistical details, *P < 0.05, n = 9–10 per group).
levels. This general pattern was evident in both H and NH groups.

3. Discussion

The findings of Exp. 1 indicate two things. Firstly, preventing mesocortical DA (released during stress) from acting on its receptors in the IL cortex causes an exaggerated neuroendocrine stress response to restraint. Secondly, this DAergic modulation is lateralized to the right hemisphere as a function of early postnatal H stimulation.

On the former point, it thus appears that, under normal conditions, the net effect of DAergic actions within the IL cortex during stress is to prevent the otherwise excessive activation of the HPA axis and facilitate the return to normal circulating levels of glucocorticoids following the termination of the stress. In other words, DA in the IL cortex acts in a compensatory, negative feedback capacity rather than contributing to HPA activation. These findings are somewhat at odds with a recent report that, when DA receptor antagonists (particularly D1) were retrodialyzed into the medial PFC, the ACTH response to interleukin-1β injection was suppressed rather than enhanced (Spencer et al., 2004). In the same study, however, the ACTH response to air puff stress tended to be exaggerated by the same antagonists. A possible reason for the apparent discrepancy with our results is that interleukin-1β injection and restraint stress are qualitatively very different stressors. Furthermore, the fact that all drug infusions in the cited study were into the left medial PFC may be a relevant consideration in accounting for some of these apparent differences. What such studies make clear, however, is that the role of the medial PFC itself, and of its afferent DAergic modulation, varies with different stressors and the distinct neural circuits activated by each (Crane et al., 2003; Diorio et al., 1993; Figueiredo et al., 2003; Herman et al., 1996; Spencer et al., 2004). Moreover, the application of D1- and D2-type antagonists separately may yield different effects than blocking the two simultaneously to more completely suppress the net endogenous DAergic modulation, as with α-flupenthixol.

Fig. 5 – Effects of repeated (5) restraint stress on plasma CORT in NH (above) and H (below) rats. As with ACTH, H animals tended to habituate and NH animals to sensitize to repeated stress exposure (see text for details, *P < 0.05, n = 9–10 per group).

Fig. 6 – Effect of repeated (5) restraint stress on post mortem levels of DA (above) and DOPAC (below) in the IL cortex of NH and H rats. While levels of DA were nonlateralized in NH rats and unchanged by repeated stress, H rats showed a nonsignificant rightward shift in tissue DA content with repeated stress experience. A similar pattern was seen for the metabolite DOPAC and reflected in a highly significant shift to right cortical metabolism following repeated restraint in H rats. In marked contrast, NH rats significantly increased metabolite levels in the left IL cortex with repeated stress exposure (see text for statistical details, *P < 0.05, n = 9–10 per group).
This principle also appears evident in behavioral studies which have reported anxiolytic effects of D1 receptor agonists (Wall et al., 2004), D2 agonists and antagonists (Wall et al., 2003) and D4 receptor antagonists (Shah et al., 2004).

The present results are consistent with earlier suggestions that mesocortical DA activation represents a high level coping strategy to protect against the pathological effects of stress as medial PFC DA depletion with 6-OHDA increased the formation of gastric stress ulcer pathology, an autonomically mediated reaction to stress (Sullivan and Szechtmnan, 1995).

As well, DA-depleting lesions in the medial PFC increase anxiety-like behavior in the elevated plus maze (Espejo, 1999) and reduce social interaction (Fernandez-Espejo, 2003), suggesting an anxiolytic role for this system. Mesocortical DA activation has sometimes been proposed to be anxiogenic in nature, on the grounds that benzodiazepines or chronic antidepressant treatments reduce activity of this system and that reducing PFC DA activity should be a therapeutic target in stress-related psychopathology (e.g., Dazzi et al., 2001a,b). However, there is much direct evidence to support the role of this system as being an adaptive or protective one, in coping with stressful challenges, as has recently been reviewed in greater detail (Sullivan, 2004).

It is interesting to note that the ability of DA blockade to increase ACTH levels (Fig. 2) was virtually immediate, especially in NH rats which are generally regarded as being more sensitive to stress (Levine, 1957; Meaney et al., 1989, 1996). It should be pointed out that the first blood sample (time 0 in the restrainer) was preceded by the intracranial injection and the arousal inherent in the handling and injection procedure would have begun to activate the HPA axis during this 4- to 5-min interval. Indeed, the levels of stress hormones observed at this time were necessarily somewhat higher across all groups than the “baseline” values we normally observe with the restraint procedure alone (as in the pre-stress values of the acute restraint rats of Exp. 2, see Fig. 4). As such, the DA blocker would have had at least a few minutes to exert its effects on the initial activation of IL output neurons (essentially disinhibiting them) and further enhancing HPA activation. Given that the release of ACTH (pituitary) is upstream from that of CORT (adrenal cortex), it is not surprising that the drug effect on the former occurred earlier than those of the latter. It is somewhat surprising, however, that the effects on CORT were not evident until the post-stress period and not at the 20 min point. The reason for this remains unclear but may be related to the delayed actual peak in CORT levels, which may have been still rising at 20 min (Diorio et al., 1993).

An additional point concerns the pharmacological specificity of α-flupenthixol. While it was chosen because of its high affinity for both D1- and D2-type receptors, it should be noted that serotonin (5HT-2A and 2C) receptors would also have been affected. We subsequently injected separate groups of standard-reared rats in the left or right IL cortex with 10 μg of the SHT-2A,C antagonist ketanserin using the same injection and restraint procedure and found no effects on ACTH or CORT (data not shown). Nevertheless, one should not rule out the possibility that serotonin (or serotonin–DA interactions) could yet be playing some role, not only in the present results, but also in the prefrontal modulation of stress physiology generally.

The data also highlight the importance of early environmental rearing in the cortical regulation of HPA function. While unilateral infralimbic DA blockade in either the left or right PFC was effective in disinhibiting HPA activity in NH rats, only right-sided blockade was effective in H animals. Such results parallel earlier findings by Denenberg (1981) using large unilateral cortical lesions, where the regulation of emotion-related behaviors was nonlateralized in NH rats but was controlled by the right cerebral cortex in H rats (with the left cortex actually exerting a reciprocal, inhibitory control over the right). Using a similar postnatal handling procedure (novelty exposure), Tang and colleagues have shown a rightward shift in hippocampal volume, as well as enhanced neural plasticity (long-term potentiation) and increased sensitivity to CORT, specific to the right hippocampus of rats exposed to novelty as pups (Tang, 2003; Verstynen et al., 2001). We have also reported that, relative to NH rats, H induces a rightward shift in benzodiazepine receptor binding across prefrontal and hippocampal regions, which was negatively correlated with stress-induced neuroendocrine activation (Sullivan and Gratton, 2003). The present data indicate that the mesocortical DA system is similarly affected by early life stimulation, which appears to confer a relative increase in right-sided inhibitory tone in key limbic and cortical regions involved in stress regulation. Asymmetrical (right-sided) mesocortical DA activity has previously been associated with exposure to novel environments (Berridge et al., 1999), reduced anxiety in the elevated plus maze (Thiel and Schwarting, 2001), successful escape performance following exposure to uncontrollable

Table 1 - Correlations between DAergic measures in IL cortex and stress hormone levels

<table>
<thead>
<tr>
<th>Group</th>
<th>Variables correlated</th>
<th>r value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Handled-acute restraint</td>
<td>Left HVA with peak CORT</td>
<td>0.73</td>
<td>0.026*</td>
</tr>
<tr>
<td>(H-AR, n = 9)</td>
<td>Left DOPAC with peak CORT</td>
<td>0.54</td>
<td>0.130</td>
</tr>
<tr>
<td></td>
<td>Right DOPAC with peak CORT</td>
<td>−0.50</td>
<td>0.160</td>
</tr>
<tr>
<td>Handled-repeated restraint</td>
<td>Left DA with peak CORT</td>
<td>0.61</td>
<td>0.046*</td>
</tr>
<tr>
<td>(H-RR, n = 10)</td>
<td>Right DA with peak CORT</td>
<td>−0.72</td>
<td>0.012*</td>
</tr>
<tr>
<td></td>
<td>Left DOPAC with peak CORT</td>
<td>0.40</td>
<td>0.220</td>
</tr>
<tr>
<td></td>
<td>Right DOPAC with peak CORT</td>
<td>−0.57</td>
<td>0.070</td>
</tr>
<tr>
<td></td>
<td>Right DA with peak ACTH</td>
<td>−0.53</td>
<td>0.095</td>
</tr>
<tr>
<td></td>
<td>Left HVA with peak CORT</td>
<td>0.68</td>
<td>0.022*</td>
</tr>
<tr>
<td>Nonhandled-acute restraint</td>
<td>Right DOPAC with pre-stress CORT</td>
<td>−0.80</td>
<td>0.009*</td>
</tr>
<tr>
<td>(NH-AR, n = 9)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonhandled-repeated restraint</td>
<td>Left DOPAC with peak ACTH</td>
<td>0.53</td>
<td>0.141</td>
</tr>
<tr>
<td>(NH-RR, n = 9)</td>
<td>Right DOPAC with peak CORT</td>
<td>−0.65</td>
<td>0.057</td>
</tr>
<tr>
<td></td>
<td>Right DA with peak ACTH</td>
<td>−0.73</td>
<td>0.027*</td>
</tr>
<tr>
<td></td>
<td>Right DOPAC/DA with pre-stress ACTH</td>
<td>−0.75</td>
<td>0.020*</td>
</tr>
<tr>
<td></td>
<td>Right DOPAC with peak ACTH</td>
<td>−0.67</td>
<td>0.048*</td>
</tr>
</tbody>
</table>

DAergic measures show a close functional association with stress hormone levels, most consistently in animals with repeated stressful experience. Moreover, right-sided measures are generally negatively correlated with ACTH and CORT levels, while left-sided measures more often showed positive correlations, both in H and NH groups.

* P < 0.05.

This table presents correlations between DAergic measures and stress hormone levels, showing a close functional association with stress hormone levels, particularly in animals with repeated stressful experience. Right-sided measures are generally negatively correlated with ACTH and CORT levels, while left-sided measures more often showed positive correlations, both in H and NH groups.
shock (Carlson et al., 1993) and reduced formation of gastric stress pathology (Sullivan and Szechtman, 1995).

The results of Exp. 2 reveal that H and NH rats react differently to repeated stress, both neurochemically and in HPA axis function. Although we have previously observed H rats to show much stronger HPA axis habituation with this protocol, while NH rats remained unchanged (unpublished observations), the present Treatment x Stress interaction indicates that the HPA activation of H and NH rats did progress differently as a function of repeated restraint, with H rats tending to habituate and NH rats sensitizing. A similar pattern has been reported with more severe repeated restraint regimens (Bhatnagar and Meaney, 1995). While H/NH differences in HPA responses to repeated stress are not novel, such divergence in DAergic measures has not been reported.

On the surface, it may appear that the neurochemical profile of the H-AR rats of Exp. 2 (tending to be left-lateralized for DA and DOPAC) is not consistent with the results of Exp. 1, where only right-sided DA receptor blockade significantly affected HPA function in H rats. However, this may not be so surprising since, as stated above, early H stimulation induces a right-lateralized cortical specialization in regulating emotion-related processes (Deningberg, 1981). If the cortical regions presently examined also develop an intrinsically right-lateralized control of stress-related hormonal regulation as a function of H, then it would be expected that pharmacological manipulations in the right cortex would be most effective in altering such measures in H rats, irrespective of subtle hemispheric differences in neurotransmitter tissue content.

The most striking of the present findings is the extent to which H rats shift to a rightward bias in IL cortex DA metabolism with repeated stress experience. The correlational analyses (within groups) suggest that levels of DA and DOPAC in the right IL cortex were specifically associated with reductions in the levels of stress hormones, most notably after repeated restraint. Interestingly, this relationship was true for both H and NH rats, yet from Fig. 6, it is clear that H rats are far more capable of enhancing right mesocortical DA function following repeated stress than are NH rats, which, in fact, go in exactly the opposite and presumably less adaptive direction. This differential ability to upregulate right mesocortical DA function may thus be causally related to the divergence in HPA activity between H and NH rats over the course of stressful experience.

The present findings employing post mortem tissue measures cannot directly assess mechanistic differences in DA release, reuptake, synthesis, etc. While a number of such factors might explain the observed results, a reasonable possibility is that increased levels of DOPAC reflect higher levels of released DA available for metabolism, while higher levels of tissue DA may be reflective of enhanced synthesis; in other words, a generally upregulated system. A recent voltammetry study confirmed that stress-induced extracellular DA release in the IL cortex varies as a function of early maternal care by comparing the offspring of High and Low Licking/Grooming Dams (Zhang et al., 2005), which in many aspects parallel H and NH rats, respectively. It was found that Low rats showed a significant blunting of the extracellular DA stress response in the right IL cortex and an exaggerated response on the left side over three daily 15-min sessions of tail pinch stress. The High offspring, however, did not show the type of right-lateralized shift over days of testing like the H rats of the present study, perhaps owing to the different nature of the stressors, as rats may be less likely to habituate or adapt to tail pinch stress than simple restraint.

Lesions of the medial FPC/anterior cingulate have been shown to disrupt the normal process of stress adaptation, reflected in gastric stress pathology in a more chronic repeated restraint paradigm (Sullivan and Henke, 1986). It has also been shown in lesion studies in standard-reared rats that the medial PFC regulation of HPA axis function becomes increasingly (right) lateralized with repeated restraint compared to acute restraint (using the same repeated restraint protocol presently employed; Sullivan and Gratton, 1999). The present data suggest that the DAergic modulation of this intrinsically asymmetrical process follows a similar trajectory over the course of repeated stress exposure, at least in rats receiving a critical level of early postnatal stimulation. The data also highlight the right IL cortex as a critical site in mediating the adaptive incorporation of stress-related experience. This too may not be surprising given the importance of an optimal window of prefrontal DAergic function in numerous forms of learning and working memory, not only in primates (Sawaguchi and Goldman-Rakic, 1991; Williams and Goldman-Rakic, 1995), but also in various attentional and learning processes in rodents, including the extinction of conditioned fear (Fernandez-Espejo, 2003; Granon et al., 2000; Pezze et al., 2003; Robbins, 2000).

As mentioned, early H stimulation is associated with highly efficient HPA axis function and enhanced feedback regulation at various levels (Caldji et al., 1998; Levine, 1957; Meaney et al., 1989, 1996). It is thus possible that such optimal stress regulation may be at least partially dependent upon the changes in cortical development triggered by the H treatment, namely, the right hemispheric specialization in regulating stress- and emotion-related processes. Moreover, the mesocortical DA system appears to be a key player in such optimal stress regulation and neuroendocrine control. These findings also raise the possibility that deficiencies in mesocortical DA function may in part be responsible for the excessive or dysregulated HPA activity seen in stress-related psychiatric conditions like major depression and anxiety disorders. Such deficiencies could also be significantly related to the lateralized imbalances in prefrontal function, so frequently seen in such disorders (for review, see Sullivan and Gratton, 2002b). Finally, potential treatments which are most effective in gradually enhancing rather than reducing mesocortical DA activity may be of particular therapeutic value in conditions of heightened stress sensitivity and impaired coping ability.

4. Experimental procedures

4.1. Exp. 1—DA receptor blockade in IL cortex and HPA activation in response to acute restraint

All procedures were in accord with Canadian Council on Animal Care guidelines and were approved by the local hospital (Louis-H. Lafontaine) Research Ethics Committee.
Pregnant Long–Evans rats (Charles River, St. Constant, Quebec) were acclimated to our animal facility for the final 7–10 days of gestation. Following birth, litters were culled to 10 pups/litter, keeping roughly equal numbers of each sex. From postnatal days 1–14, half of the litters received a daily handling (H) treatment as follows. First, the mom was placed alone in an adjacent novel cage with regular bedding. Pups were then removed from the homecage and placed together in a novel cage lined with paper towel for 15 min. The pups and then mom were returned to the homecage. Upon return to the homecage, beneficial maternal behaviors are known to be stimulated, and this was confirmed by our own observations. In particular, licking/grooming and arch-backed nursing are increased, behaviors which correlate with reduced stress reactivity in offspring tested as adults (Caldji et al., 1998).

The other half of the litters were left completely undisturbed or nonhandled (NH), save for a single cage cleaning on day 7, reduced from the standard bieweekly cage maintenance. Rats were weaned on day 21 and housed in pairs until testing as adults. Due to practical (space) considerations, only males were used for the adult studies. It should be noted that brief separations of the mom and pups, as in the H rats, closely mimic the natural species conditions where the moms must leave the nest for brief periods to forage. As such, H may approximate more normal or optimal conditions where the moms must leave the nest for brief periods.

4.2. Postnatal handling procedures

Starting at 70 days of age, rats of each treatment condition (H or NH) were randomly assigned to one of three subgroups (Sham, Left or Right, with a final n of 10–12 for each of the six groups). Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) following atropine pretreatment (0.1 mg/kg, i.p.) and implanted unilaterally with a 26-gauge guide cannula just above the IL cortex following acute and repeated restraint (3 h), which are known to have detrimental long-term effects (e.g., Liu et al., 2000).

4.3. Surgery, injections and stress testing

Starting at 70 days of age, rats of each treatment condition (H or NH) were randomly assigned to one of three subgroups (Sham, Left or Right, with a final n of 10–12 for each of the six groups). Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) following atropine pretreatment (0.1 mg/kg, i.p.) and implanted unilaterally with a 26-gauge guide cannula just above the IL cortex (AP +3.0 mm, L ±0.8, V –3.7). Shams (vehicle injected) were implanted alternately in the left or right IL cortex.

A week later, all animals were restrained in Plexiglas large rodent restrainers (Harvard) for 20 min at room temperature with blood samples (125–150 μl) collected quickly from the tip of the tail in eppendorf tubes containing trasyol and EDTA. Samples were taken at 0, 20 and 80 min from stress onset, corresponding roughly to pre-stress, peak stress and stress recovery time points. Blood was centrifuged at 2200 rpm for 15 min and plasma frozen at –80 °C. Immediately prior to placement in restrainers, rats were injected intracranially via a 33-gauge injection cannula projecting 1 mm beyond the tip of the guide into the IL cortex and attached to a Hamilton microsyringe with PE30 tubing. Rats were injected over a 2-min period (with an additional 2 min for diffusion) with 1 μl of either artificial cerebrospinal fluid (aCSF) or α-flupentixol (20 μg, Sigma). This single relatively large dose was intended to negate the effects of the pronounced stress-induced DA release on IL output neurons by locally blocking both D1- and D2-type DA receptors. This dose was chosen since 20 or 25 μg injected bilaterally in medial PFC was shown to alter conditioned fear or freezing behavior in rats without altering general activity (Pezze et al., 2003). Pilot injections of dye confirmed that the injected volume diffused throughout, but not beyond, the ipsilateral IL and ventral prelimbic cortex. As such, any observed effects are presumably due to actions on DA receptors throughout these subfields of ventromedial PFC, although we refer to IL cortex as the predominant site of action. Moreover, the pronounced stress-induced DA release is significantly more pronounced in IL cortex than more dorsal (prelimbic) sites (Doherty and Gratton, 1996). No bilateral injection groups were included in this study as we have previously shown that, regarding the medial PFC modulation of stress and emotionality, unilateral lesion effects (when present) are at least as pronounced as those of bilateral lesions (Sullivan and Gratton, 1999, 2002a; Sullivan and Szechtman, 1995), and a focus of this study was to examine the two hemispheres separately.

At the end of testing, rats were given an overdose of urethane, perfused intracardially with 0.9% saline and 10% formalin and brains removed. Frozen sections (20 μm) were later stained with thionin to confirm injection sites. Frozen plasma samples were later analyzed for levels of corticosterone (CORT) and adrenocorticotropic hormone (ACTH) using radioimmunoassay kits (Medicor). The limits of detection in the assays for CORT and ACTH were 0.77 μg/dl and 5.7 pg/ml, respectively, both well below the range of the present samples. Stress hormone profiles were analyzed statistically using a Treatment (H vs. NH) × Group (Sham, Left, Right) ANOVA with repeated measures on Sample (0, 20, 80 min). Post hoc comparisons (Student–Newman–Keuls tests) were performed between individual groups following significant interaction effects (p < 0.05).

4.4. Exp. 2—Post mortem neurochemical analysis of IL cortex following acute and repeated restraint

H and NH rats were generated exactly as described in Exp. 1, with males being used for the adult studies. Between 60 and 70 days of age, rat behavior was videotaped for 10 min in a holeboard apparatus (100 × 100 × 50 cm) with a raised floor containing 9 equidistant holes (5 cm). The total number of holes actively investigated was recorded. The purpose of this screening test was twofold. First, this served as a behavioral confirmation of the effectiveness of the H procedure as H rats are known to explore novel environments more freely than NH rats. In our hands, this treatment typically results in approximately a third greater exploratory behavior relative to NH rats. In the present study, H rats investigated 34.5% more holes than NH rats (48.7 vs. 36.2, p < 0.05), confirming the expected behavioral differences this treatment should produce. Second, these data on individual behavioral differences provided the opportunity to rank animals within each treatment (H/NH) condition from lowest to highest exploration, after which they were alternately assigned to the subgroups described below. While this latter step was not essential, it provided an additional level of control that subgroups within each treatment were equivalent from a behavioral vantage point. The two subgroups in each treatment were designated as acute restraint (AR) or repeated restraint (RR) stress conditions (n = 9–10 for each of the four groups).

Between 70 and 90 days of age, H and NH rats in the acute restraint groups underwent a single restraint stress as described above, with the same blood sampling procedure. Rats in the repeated restraint groups underwent the same restraint procedure daily for five consecutive days, with blood sampling only on the fifth day. All restraint stress and blood collection in both experiments were conducted between 10:00 AM and 2:00 PM.

All rats were sacrificed 2–4 h following the last blood sampling (3–5 h post-restraint). The purpose of this timing was to allow the animals to return to a more baseline state following stress exposure, both in terms of stress hormone levels and neurotransmitter function (e.g., Imperato et al., 1992, 1993; Puglisi-Allegra et al., 1991). Rats were not sacrificed for neurochemical analysis immediately following restraint because we wished to obtain an important post-stress (1 h recovery) blood sample. Thus, by waiting at least 3 h after restraint, this post mortem neurochemical “snapshot” is more a reflection of general differences between H and NH animals and the effects of repeated stress experience rather than neurochemical activity during the experience of stress per se.

Rats were decapitated following rapid halothane anesthesia and brains quickly removed and dissected on ice. Tissue samples were prepared for microdialysis experiments as follows:
were taken separately from the left and right IL cortex and frozen at −80 °C for future analysis.

4.5. **High performance liquid chromatography (HPLC)**

Brain tissue samples were thawed and homogenized in artificial CSF and centrifuged at 4000 rpm for 15 min. Supernatants were filtered using a 0.45 μm syringe filter, and 5 μl was used for HPLC analysis. The pellet was used for protein assay using the Bradford protein assay technique, such that neurotransmitter and metabolite levels are expressed as pg/mg protein.

Samples were injected into a 20 μl sample loop with a rhenodeyne injector (model 7125). Samples were passed over a C18 Haisil column (5 μm, 150 mm × 4.6 mm) to an electrochemical detector (ESA Coulochem, model 5100A). Electrode potentials were set for +400 mV and −350 mV, for E1 and E2, respectively. Each liter of mobile phase consisted of 5.7 g citric acid, 38 mg EDTA, 8.27 g sodium phosphate monobasic, 24 mg sodium dodecyl sulfate and 120 ml acetonitrile, with a pH of 3.7. Flow rate was 1.2 ml per minute. Data were analyzed using EZ Chrom software.

The compounds which could be reliably quantified with this protocol included DA, its major metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), as well as norepinephrine (NE) and the serotonin metabolite 5-hydroxyindole acetic acid (5-HIAA).

Neurochemical measures were analyzed statistically using a Treatment (H vs. NH) × Stress (acute vs. repeated) ANOVA, with repeated measures on the within subjects factor of Side (left vs. right). Post hoc comparisons (Student–Newman–Keuls tests) were performed between individual groups following significant interaction effects (α 0.05). As well, correlations between neurochemical measures and stress hormone levels within individual groups were performed using Pearson’s test of correlation coefficients.

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