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Brain Research 826 (1999) 35–43

BRAIN
RESEARCH

Research report

Activation of serotonin-immunoreactive cells in the dorsal raphe nucleus in rats exposed to an uncontrollable stressor

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Accepted 2 February 1999

Abstract

The dorsal raphe nucleus (DRN) and its serotonergic terminal regions have been suggested to be part of the neural substrate by which exposure to uncontrollable stressors produces poor escape responding and enhanced conditioned fear expression. Such stressor exposure is thought to selectively activate DRN serotonergic neurons in such a way as to render them transiently sensitized to further input. As a result of this sensitized state, behavioral testing procedures are thought to cause excess serotonergic activity in brain regions that control these behaviors. The present studies were conducted to investigate activity in the DRN following exposure to escapable and yoked, inescapable tailshock. Neural activity was characterized using immunohistochemistry to detect the immediate early gene product Fos in serotonin-immunoreactive cells in the DRN. Inescapable tailshock led to greater serotonergic neural activity than did escapable tailshock, supporting the hypothesis that uncontrollable stressors preferentially activate serotonergic neurons in the DRN. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Immunohistochemistry; Serotonin; Raphe nuclei; Fos; Stress; Anxiety; Depression

1. Introduction

The nature of the physiological and behavioral reactions to an aversive event depends on whether or not the organism can control the event. Reactions that only occur subsequent to uncontrollable events but not to controllable events are referred to as learned helplessness effects. Learned helplessness, in the form of impaired escape performance, was initially demonstrated in dogs that were given a series of inescapable shocks [62,71].

Extensive characterization of learned helplessness in rodents has revealed that the effects of uncontrollable stressors, relative to controllable stressors, extend beyond escape responding to affect a broad constellation of behaviors. Other defensive behaviors that are affected by uncontrollable stressors include expression of conditioned fear [48,59], defensive burying [87], defeat posturing [88] and territorial defense [86]. Basic physiological processes, such as temperature regulation [40], eating [14], and sleeping [1] have also been shown to be sensitive to the effects of uncontrollable stressor exposure. Subjects exposed to uncontrollable stressors show exaggerated reactions to amphetamine [47] and morphine [27,34]. Altered pain sensitivity following exposure to uncontrollable stressors has been studied extensively [18–20,38,51,52,57]. Finally, uncontrollable stressors produce increased anxiety, observed as decreased social interaction in rats [74].

The behavioral effects of a particular uncontrollable stressor, inescapable tailshock (IS), have been extensively characterized (see Maier for a review [49]). Recent efforts to understand the mechanisms which mediate these phenomena have focused on serotonergic (5-HT) neurons in the dorsal raphe nucleus (DRN). The DRN provides a large portion of the 5-HT projections ascending to cortex, hippocampus, hypothalamus, other forebrain and midbrain structures [79,83]. In addition, single neurons project to the cortex and spinal cord [41]. The ubiquitous nature of DRN projections provides a framework for 5-HT influence over the widespread effects of IS on behavior. 5-HT is known to modulate escape behavior [25], sleep [39], circadian rhythmicity [2], feeding [16], anxiety [37], and pain sensitivity [84]. The proximal brain sites that mediate these behaviors all receive projections from the DRN [83]. Activation of the DRN is sufficient to blunt pain sensitivity

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PII: S0006-8993(99)01208-1

and release of 5-HT in DRN projection regions (i.e., nucleus raphe magnus) reduces pain sensitivity [36]. In addition, activation of the DRN reduces activity in the dorsolateral periaqueductal gray, an area known to mediate escape responding [44]. Indeed, 5-HT injected into this region impairs escape behavior [25]. In addition, the DRN projects to the amygdala [35,45] where 5-HT can increase neural activity [11] and produce anxious behavior [24]. Given that activity in the amygdala is necessary for fear conditioning [13], increased activity in this area would be expected to enhance fear conditioning. In addition, behaviors that are considered indices of anxiety can be directly modulated by DRN manipulations [29–31].

The foregoing suggests that activity in 5-HT neurons within the DRN could produce a constellation of outcomes similar to those produced by IS. Since behavioral testing typically occurs 24 h after exposure to IS, these behaviors would occur if IS sensitized DRN 5-HT neurons for a period of time so that they would respond in exaggerated fashion to input induced by the behavioral testing conditions (e.g., footshock during escape testing). Consistent with this possibility, lesion of the DRN and pharmacological inhibition of DRN 5-HT activity before behavioral testing reverses the effects of IS on behavior [50,53,56].

IS could sensitize DRN 5-HT neurons via a number of mechanisms but all assume that IS selectively activates DRN 5-HT neurons relative to ES [49]. Pharmacological data support this view. Pharmacological inhibition of DRN 5-HT activity at the time of IS prevents learned helplessness effects [55,56,81], and pharmacological activation of these neurons in the absence of IS produces behavioral sequelae similar to those of IS [50,81]. These studies suggest, but do not provide direct evidence, that IS selectively activates DRN 5-HT neurons.

Fos immunohistochemistry has become a popular technique to assay neural activity [17]. The protein Fos is the product of the immediate early gene c-*fos* and is one of many transcription factors found in most cells [61]. Transcription factors bind to DNA sequences to modulate transcription of genes. The same sequence can occur on many genes, allowing the induction of a transcription factor to orchestrate the transcription of a number of genes in response to demands made on the cell. Since the immediate early gene products are thought to mediate the metabolic challenge imposed on the cell by strong activation [61], Fos and other products of these genes are considered ‘third-messenger’ cellular signals [60].

The use of Fos as a marker for DRN neural activation following IS is warranted for several reasons. Serotonin cells can be identified and characterized as being activated using double-labeling in which nuclear Fos and cytoplasmic serotonin can be visualized. The cellular signals known to induce Fos, such as the second messenger cAMP [32], are known to occur in serotonin neurons in the DRN. The signals proposed to activate the DRN as a result of IS have all been shown to induce Fos in other brain regions. For example, NMDA receptor activation is well characterized as an inducer of Fos [76] and is also involved in the DRN-dependent effects of IS (Grahn et al., unpublished data). Other signals known to induce Fos expression, including opioid-receptor activation [9,23], interference with GABA inhibition [69], and activation of noradrenergic receptors [5,80] are thought to be processed by the DRN during IS [26,50,54,56]. All of these signals have been shown to change levels of Fos in various brain regions, although the DRN has not been the focus of such investigations.

Surprisingly, given the extent to which Fos has been employed as an activation marker, only a handful of reports have demonstrated double-labeling of serotonin and Fos in the DRN [15,43,46,90]. The neural circuitry activated during various stressors has been studied extensively using Fos. These studies have focused on brain sites that are traditionally considered to be involved in the effects of stressors, such as the components which activate the HPA axis (hippocampus, cortex, hypothalamus) [12,65,70,89] or sites included in ‘fear’ circuitry (amygdala, cortex, septum, periaqueductal gray) [7,10,21,75], and lower brainstem regions (locus coeruleus, nucleus tractus solitarius, ventrolateral medulla) [8,65,67,75]. When the DRN has been examined, it has been reported to have very little basal Fos expression. Some, but not all stressors, increase Fos expression in the DRN [3,4,42,72].

The aim of the present studies was to investigate a neural consequence of exposure to stressor conditions that are identical to those that are known to cause learned helplessness behavior. Recently, IS was shown to produce a large accumulation of extracellular 5-HT within the DRN relative to ES [58]. However, the release of serotonin within the DRN has been shown to occur from dendrites and soma and can be independent of depolarization [82]. Release of serotonin is also modulated by activation of somatodendritic and terminal autoreceptors in such a way that neural activation and release do not always correlate [66]. Further, it is possible that serotonin released in the DRN at the time of IS originates in other raphe nuclei which project to the DRN [78]. The present studies therefore employed the activation marker Fos to characterize DRN activity following IS or ES. Double-labeling of neurons for serotonin and Fos was conducted to assess activation specifically in serotonin neurons.

2. Materials and methods

Male Sprague–Dawley rats, 80–100 days old, served as subjects in all experiments. They were housed individually with free access to standard lab chow and water. All procedures were in accordance with NIH animal care guidelines and were conducted with approval of the University of Colorado Institutional Animal Care and Use Committee.

2.1. Stressor exposure

Rats were exposed to a single 2 h session of either ES or IS in wheel-turn chambers measuring 14 × 11 × 17 cm. Pilot studies indicated that there was no basal 5-HT neural activation so a non-stress group was not included. The rat's tail protruded from the rear of the chamber and was secured with tape to a bar, with electrodes fixed to the distal portion of the tail. The opposite side of the chamber was equipped with a wheel measuring 9 cm in diameter that could be turned by escape subjects but was rendered immobile for yoked subjects. Tailshock consisted of 100 1.0 mA shocks with an average intertrial interval of 60 s. Shock duration was determined by the response latency of subjects in the escape condition and was automatically terminated at 30 s if no response occurred. Each shock was terminated for both the escape and yoked subject when the escape subject responded. The escape subject's responding was monitored automatically and the difficulty of the task (number of wheel revolutions required for shock termination) was increased gradually until consistent responding was attained. During the initial training period, the response requirement was a single quarter turn of the wheel, considered a single unit of responding. Three consecutive response latencies shorter than 5 s were followed by an increase in the task difficulty by a single unit (to equal a required 1/2 wheel turn). Subsequent responses that were performed under 5 s resulted in the requirement being doubled ($1 \rightarrow 2$, $2 \rightarrow 4$, $4 \rightarrow 8$ etc.) until the maximum response requirement of 16 units of responding was achieved. In instances in which no response was performed, the criteria was relaxed to a single quarter turn. Response latencies over 5 s resulted in the response requirement to decrease by one unit. Thus, each pair, including an ES-treated and an IS-treated subject, received the same pattern, number, intensity and duration of shocks.

2.2. Tissue preparation

Rats were deeply anesthetized with sodium pentobarbital (Nembutal) approximately 1, 2, 3, or 4 h after the last tailshock. Rats were perfused transcardially with 50 ml physiological saline for 30 s followed by 400–500 ml of 4% paraformaldehyde in 0.1 M phosphate buffer. Extracted brains were post-fixed in the same 4% paraformaldehyde overnight and then transferred to 30% sucrose until sectioning. Sections measuring 25 μm were obtained in a −20°C cryostat and were stored at 4°C in cryoprotectant.

2.3. Immunohistochemistry

Immunohistochemical staining for 5-HT and Fos were conducted sequentially. Sections from each subject were chosen to represent the rostral to caudal extent of the DRN. Staining for Fos was conducted first using the avidin-biotin-horseradish peroxidase (ABC) method. Sections were washed three times for 10 min each in phosphate buffered saline (PBS) prior to a 24 h incubation with a 1:15,000 dilution of Fos primary antibody (Santa Cruz Lot #J125) in a blocking solution containing 1% normal goat serum, 1% bovine serum albumin, 0.25% Triton-X, and 0.1% sodium azide. This incubation was followed by another series of washes in PBS after which sections were incubated for 2 h in biotinylated goat anti-rabbit secondary antibody (Jackson Laboratories) diluted 1:200 in blocking solution. Following a series of washes in PBS, sections were incubated with avidin-biotin-horseradish peroxidase complexes (Vectastain Elite ABC kit) in PBS for 1 h. After a series of washes with phosphate buffer (PB), sections were exposed to a solution containing diaminobenzidine (DAB), cobalt chloride, ammonium chloride, nickel ammonium sulfate and glucose oxidase in phosphate buffer for 10 min. The peroxidase reaction was initiated by addition of glucose solution and reacted for approximately 15 min. The reaction was terminated by placing sections in PBS.

5-HT staining was conducted using the peroxidase anti-peroxidase (PAP) method. Sections were washed in PBS as before. Excess background 5-HT staining was prevented by incubating sections in blocking solution for 0.5 h. Tissue was then exposed to blocking solution containing a 1:10,000 dilution of 5-HT antibody (Incstar Lot #516225) for 48 h at 4°C. Goat anti-rabbit secondary antibody (1:200) was applied to the sections for 2 h after a series of washes in PBS. This step was followed by another series of PBS washes after which sections were incubated with a 1:500 dilution of PAP antibody complexes (Sigma Lot #116H4847) for 2 h. Following a series of washes in PB, tissue was incubated in a solution containing DAB and glucose oxidase. The peroxidase reaction was initiated by addition of glucose and continued for approximately 15 min. After a series of washes sections were mounted on slides which were allowed to dry overnight. Slide-mounted sections were dehydrated in a series of alcohols and defatted with Histoclear. Slides were coverslipped with Permount.

2.4. Image analysis

Sections were analyzed by an observer blind to the experimental conditions. Each of three sections for each rat was assessed for the number of Fos-stained nuclei, the number of 5-HT-stained cells, and the number of double-labeled cells. Ovoid particles that were dark-brown or black were counted as Fos-stained nuclei. Larger tan particles, with and without obvious unstained nuclei, were counted as 5-HT-positive cells. Sections were chosen to represent the rostral to caudal extent of the DRN. Rostral sections were comparable to an anterior–posterior coordinate of −1.36 mm from interaural zero according to

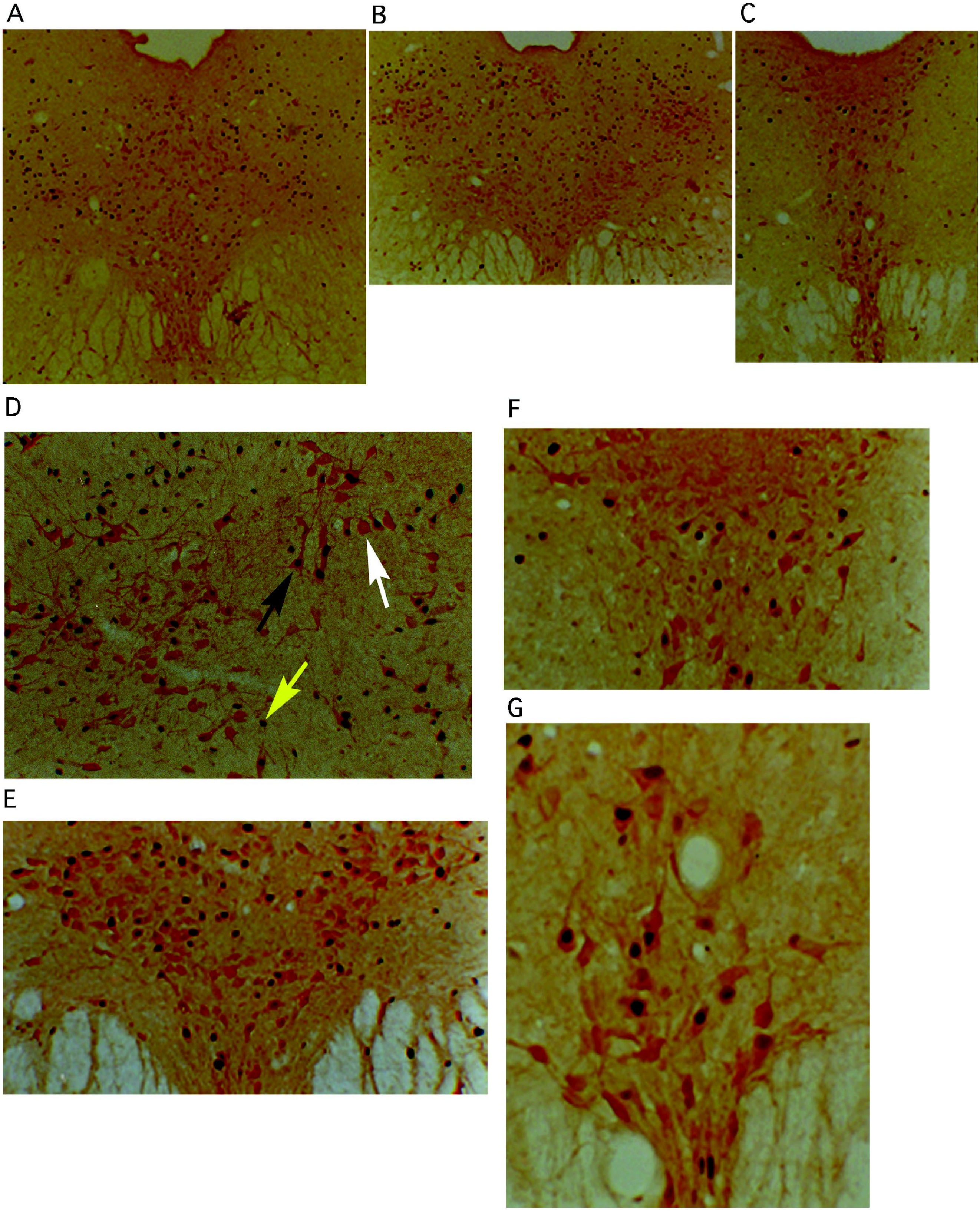

Fig. 1. Photomicrographs were taken of the DRN from a rat exposed to inescapable tailshock and sacrificed 2 h after the end of the shock session. Panels A, B, and C show representative sections of rostral, middle, and caudal sections of the DRN analyzed for each subject. Panel D shows the dorsolateral region of a middle DRN section. The yellow arrow denotes a Fos-immunoreactive nucleus, the white arrow points to a 5-HT-immunoreactive cell, and the black arrow identifies a double-labeled cell. The ventral region of a middle DRN section is shown in Panel E, while the dorsal and ventral regions of a caudal section of DRN are shown in Panels F and G, respectively.

Table 1
Adult male rats were exposed to escapable tailshock or to the same amount of inescapable tailshock. Brain tissue was processed 1–4 h after stressor exposure. The number of Fos-immunoreactive nuclei and the number of 5-HT-immunoreactive cells were determined in three regions of the DRN

Time after shock session	DRN region	Fos-positive nuclei						5-HT-positive cells					
		Escapable shock			Inescapable shock			Escapable shock			Inescapable shock		
		n	Mean	S.E.M.	*n*	Mean	S.E.M.	*n*	Mean	S.E.M.	*n*	Mean	S.E.M.
1 h	Rostral	5	42.80	13.10	6	61.17	6.64	5	81.40	6.06	6	80.33	5.60
	Middle	5	70.80	21.03	6	103.67	7.75	5	123.40	5.45	6	114.50	4.55
	Caudal	5	49.40	14.64	6	53.50	5.40	5	71.80	2.18	6	64.00	6.80
2 h	Rostral	6	66.00	7.67	5	71.40	6.38	6	84.17	8.50	5	81.40	3.70
	Middle	6	111.83	12.34	5	106.2	7.28	6	131.83	10.86	5	135.40	4.11
	Caudal	6	59.00	9.11	5	65.40	13.08	6	94.83	9.62	5	78.80	3.51
3 h	Rostral	8	71.25	14.26	8	107.62	16.88	8	92.50	5.25	8	94.13	8.55
	Middle	8	132.62	33.92	8	89.25	16.02	8	138.63	9.87	8	150.63	9.84
	Caudal	5	131.40	39.86	7	61.86	12.41	5	99.60	5.18	7	91.00	5.47
4 h	Rostral	6	36.83	7.07	5	46.80	2.96	6	97.67	5.54	5	108.60	9.51
	Middle	6	32.83	8.60	5	44.00	2.19	6	145.17	4.39	5	159.00	7.37
	Caudal	6	35.50	8.33	3	37.00	7.94	6	88.00	2.46	3	86.00	4.16

Paxinos and Watson [63]. The middle and caudal sections were comparable to −1.00 mm and −0.70 mm anterior–posterior coordinates, respectively.

2.5. Statistical analysis

The dependent variables were the number of Fos-stained nuclei, the number of 5-HT-stained cells, and the number of double-labeled cells. A two-way analysis of variance (ANOVA) was performed for each variable with DRN regions and stressor conditions as the independent variables. Each timepoint was treated as a separate experiment.

3. Results

Photomicrographs of the DRN examined from an IS-treated rat 2 h after stressor exposure are shown in Fig. 1.

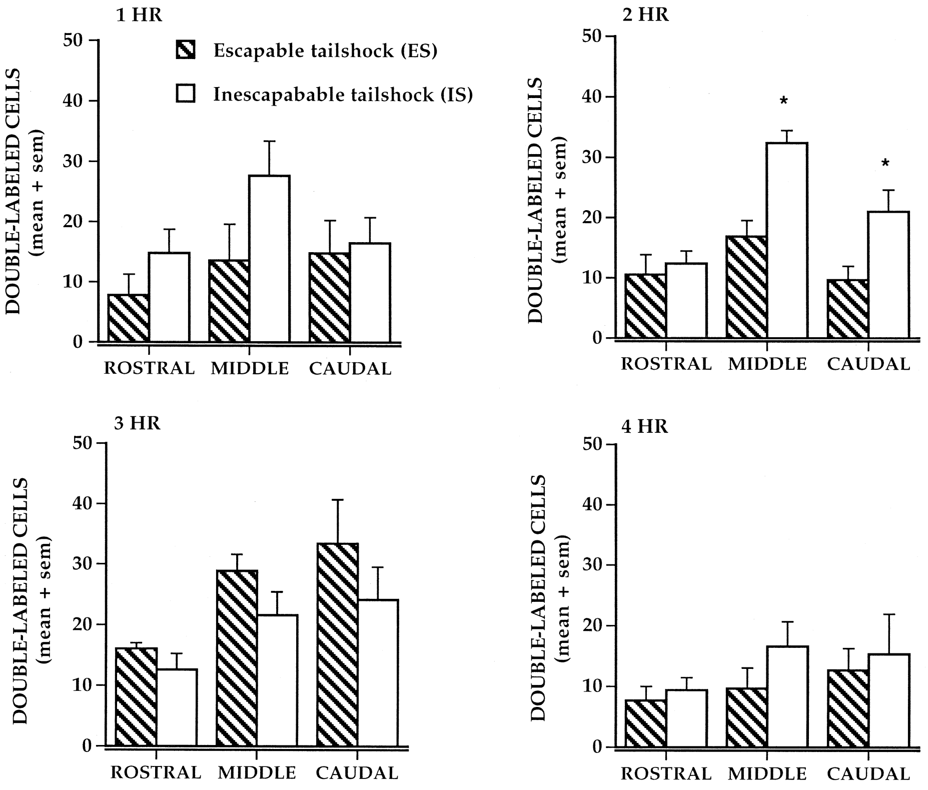

Fig. 2. Activation of serotonergic neurons in the DRN is shown as the number of cells double-labeled with serotonin and the activation marker Fos. Rats were exposed to escapable tailshock (hatched bars) or to the same amount of inescapable tailshock (open bars). Asterisks denote significant between-group differences.

Panels A–C represent the rostral, middle and caudal sections examined for each rat. Panels D–G are higher magnifications showing each type of particle analyzed. As shown in Table 1, the mean number of 5-HT-stained nuclei was not different between IS and ES conditions for any time-point. Table 1 also shows the number of Fos-stained particles observed in three regions of the DRN for each time-point. There were no significant differences in Fos-stained particles between ES- and IS-treated subjects for any time or region. Fig. 2 shows the number of double-labeled cells in three DRN regions for the four timepoints. The number of double-labeled cells observed 2 h after stressor exposure was greater after IS than after ES in the middle and caudal regions of the DRN. These observations were confirmed by a two-way ANOVA which revealed a significant effect of stressor exposure, $F(1,27) = 13.306$, $p < 0.001$, and a significant effect of DRN region, $F(2,27) = 8.811$, $p < 0.001$. Orthogonal contrasts between the two stressor conditions within each region indicated that differences due to IS only occurred in the middle and caudal regions of the DRN.

4. Discussion

The present results indicate that Fos expression in DRN 5-HT neurons is preferentially induced by exposure to IS, relative to ES. Fos expression in these experiments exhibited the expected time-course with maximal expression 2–3 h after IS or ES and decreased expression 4 h after stressor exposure. It is notable that there would be no controllability effect if expression of Fos alone were examined. As shown in Table 1, Fos expression 2 h after stressor exposure was similar in both IS- and ES-treated subjects. However, the IS-treated subjects had a higher number of double-labeled cells, suggesting that Fos was being expressed in non-5-HT cells in the ES-treated subjects. It is interesting that the DRN sensitization hypothesis of learned helplessness [49] would expect more activity in cells that release GABA in the ES condition.

Differences due to stressor controllability were only evident in the middle and caudal regions of the DRN. This observation is germane to the microinjection studies described above in which drugs that altered DRN 5-HT activity were administered into the DRN. The cannula implantation procedures used in these studies generally result in caudal DRN injection sites. The DRN has been described as containing subnuclei which have unique projections and functions. Caudal regions of the DRN have been shown to project to areas including the hippocampus and locus coeruleus [35], as well as the amygdala [68] and dorsolateral periaqueductal gray [44]. In addition, ascending fibers from various brain regions terminate in specific subregions of the DRN [64]. The double-labeling results obtained in the present study may guide future efforts to elucidate the source of DRN activation during IS.

The expression of Fos in the DRN can be viewed in relation to the intracellular mechanisms by which the c-*fos* gene is induced. The c-*fos* gene contains a DNA binding sequence in its regulatory region that recognizes the transcription factor cAMP/Ca^{2+} regulatory element binding protein (CREB) [73]. CREB is produced by activation of calmodulin which is produced by increased levels of Ca^{2+}. Activation of 5-HT neurons causes the increased cAMP production [6] that could initiate this cascade leading to CREB activation. It is interesting to note that 3 h after stressor exposure there was a trend toward a shift to a slightly higher number of double-labeled neurons in the ES condition. It is possible that this reversal reflects activation of somatodendritic autoreceptors in the IS condition. Activation of these receptors has been shown to decrease adenylyl cyclase activity thereby reducing levels of cAMP [6]. This reduction in cAMP would be reflected in decreased induction of the c-*fos* gene. This slight decrease could also reflect the negative modulation that Fos exerts on its own expression [32].

While these studies have exploited Fos as a marker of neural activation, the results warrant exploration of Fos-regulated gene transcription in the DRN following exposure to IS. Some genes for endogenous opioids are known to be regulated by Fos [33]. The gene that codes for glutamic acid decarboxylase may also be regulated by Fos [32]. Enkephalin and GABA [85] and GABA and serotonin [22,28,77] have been demonstrated to be colocalized in the DRN. Fos regulation of gene transcription may contribute to the DRN-dependent behavioral effects of IS.

Acknowledgements

Support was provided to SFM by NIMH MH50479 and RSA MH00314. Additional support was provided to MBM by the University of Colorado Undergraduate Research Opportunity Program. We would like to thank Jack Yamuy and Ron Gaykema for helpful advice regarding serotonin immunohistochemistry.

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