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A BRAINSTEM SUBSTRATE FOR ANALGESIA ELICITED BY INTRAORAL SUCROSE

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Abstract—Previous studies demonstrated that nursing or intraoral infusion of certain components of mother's milk (e.g. sugars and fats) produces calming and opiate receptor-dependent analgesia in newborn rats and humans. However, the neural circuitry underlying such analgesia is unknown. The aim of the present study was to specify the central pathways by which taste stimuli engage neural antinociceptive mechanisms. For this purpose, midcollicular transactions were used to investigate the role of the forebrain in analgesia elicited by intraoral infusion of 0.2 M sucrose in neonatal rats. Sucrose-induced analgesia persisted, and was enhanced, following midcollicular transection, indicating that it did not require neural circuits confined to the forebrain. Fos immunohistochemistry was used to identify brainstem neurons activated by a brief (90 s) intraoral infusion of a small volume (90 μl, 0.2M) of sucrose or a salt solution (0.1 M ammonium chloride) in 10-day-old rat pups. Compared with control groups (intact, cannula, distilled water), both sucrose and ammonium chloride induced Fos expression in the rostral nucleus tractus solitarius, the first relay in the ascending gustatory pathway. Sucrose also elicited Fos expression in several brainstem areas associated with centrally mediated analgesia, including the periaqueductal gray and the nucleus raphe magnus. Taken together, these findings demonstrate that analgesia elicited by intraoral sucrose does not require involvement of the forebrain. Intraoral sucrose activates neurons in the periaqueductal gray and nucleus raphe magnus, two key brainstem sites critically involved in descending pain modulation.

Key words: immunohistochemistry, gustatory, pain, opioid analgesia, neonate.

Intraoral infusion of microliter quantities of mother's milk and certain sweet components of milk (sucrose, fructose, glucose) produces antinociceptive and calming effects in human infants and neonatal rats (Blass and Fitzgerald, 1988; Shide and Blass, 1989 and Blass et al., 1995; Ren et al., 1997; Anseloni et al., 2002). In rats, analgesia produced by intraoral sucrose infusion is developmentally transient, peaking at about postnatal day (P) 10 and is absent by the time of weaning (approximately P21; Anseloni et al., 2002). Analgesia induced by sucrose in rats and humans develops rapidly, lasts for minutes and, in rats, is blocked by systemic injection of opioid receptor antagonists (Blass et al., 1987; Anseloni et al., 2002). Thus, the properties and mechanisms of sucrose-induced analgesia appear to be remarkably well-conserved phylogenetically and opioid-dependent. The analgesic and calming effects produced by natural stimuli, such as mother's milk, or intraoral sucrose are clinically significant. For example, sweet stimuli (e.g. sugar-coated pacifiers) have been used to manage pain and stress associated with a number of clinical manipulations (e.g. heel lance, circumcision) routinely performed in infant humans (Blass and Shah, 1995; Overgaard and Knudsen, 1999).

Despite these findings, the neural circuitry by which the gustatory stimuli engage opioid receptor-dependent analgesia is unknown. Sucrose-induced analgesia is initially triggered by stimulation of taste receptors in the oral cavity and is not due to post-ingestional mechanisms as direct stomach loading of sucrose is ineffective (Ramenghi et al., 1999). In rodents, the ascending gustatory pathway consists of orderly projections from the rostral pole (rNTS) of the nucleus tractus solitarius (NTS) to the parabrachial nucleus (PbN), and in turn, from the PbN to the parvicellular portion of the ventroposterior medial thalamic nucleus (VPMpc). The VPMpc projects to a number of cortical and subcortical forebrain areas, including agranular insular cortex, lateral hypothalamus (LHA), central nucleus of the amygdala (CNA) and bed nucleus of the stria terminalis (BNST). In addition, the gustatory region of the PbN projects directly to the CNA. Descending projections from rNTS to the intermediate and lateral parvocellular subdivisions of the medullary reticular formation and the oromotor nuclei are thought to be involved in somatic motor and visceromotor reflexes (e.g. salivation) associated with ingestion (Travers and Norgren, 1983; Halsell et al., 1993, 1996).

It is noteworthy that several gustatory areas have been reported to be involved in analgesia, including the PbN (Bester et al., 1995), insular cortex (Burkey et al., 1996), CNA (Kalivas et al., 1982; Oliveras and Besson, 1988), and LHA. Additionally, tract tracing studies have demonstrated that several gustatory areas project to two major brainstem antinociceptive sites involved in opiate-receptor-

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Abbreviations: BNST, bed nucleus of the stria terminalis; CNA, central nucleus of the amygdala; DAB, 3,3′-diaminobenzidine; DW, distilled water; EF_{50}, effective force (g) to produce a 50% response frequency; IR, immunoreactive; LC, locus coeruleus; LHA, lateral hypothalamus; LPGi, lateral paragigantocellular nucleus; NTS, nucleus tractus solitarius; P, postnatal day; PAG, periaqueductal gray; PbN, parabrachial nucleus; PBS, phosphate-buffered saline; RMg, nucleus raphe magnus; rNTS, rostral pole of nucleus tractus solitarius; RVLM, rostroventrolateral medulla; VPMpc, ventroposterior medial thalamic nucleus.

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dependent analgesia: the midbrain periaqueductal gray (PAG) and the rostroventromedial medullary region that includes nucleus raphe magnus (RMg). For example, the PbN, insular cortex, CNA, LHA, and BNST densely innervate the PAG and some of these areas directly project to RVM (Behbehani et al., 1988; Rizvi et al., 1992; Krukoff et al., 1993; Behbehani, 1995; Hermann et al., 1997). Thus, there is considerable overlap among, as well as numerous potential anatomical linkages between, components of gustatory pathway and sites known to be involved in centrally mediated analgesia.

In the present study, we used midcollicular transections as well as immunohistochemistry for the protein product of the immediate early gene *c-fos*, to identify sites involved in the analgesic effect of sucrose in neonate rats. The results of this study demonstrate that sucrose-induced analgesia is mediated by circuitry within the brainstem and does not require involvement of forebrain structures. Fos immunohistochemistry showed that infusion of small, analgesic volumes of sucrose activate brainstem neurons in several areas implicated in centrally mediated analgesia, including opiate receptor-dependent analgesia. Some of these results have been previously reported in abstract form (Anseloni et al., 1999).

EXPERIMENTAL PROCEDURES

Animals and intraoral cannulation

Male and female Sprague–Dawley rat pups (Zivic Miller Co.; Harlan, Indianapolis) at postnatal days 10–12 were housed with their mother in standard polypropylene cages until the day of the experiments. Experimental procedures were carefully conducted in order to minimize animal discomfort and the number of animals used, according to an institutionally approved protocol conforming with local and international guidelines on the ethical use of animals. P10-12 pups were used because analgesia elicited by intraoral sucrose is maximal at this postnatal age range (Anseloni et al., 2002); the day of birth was considered P0. Pups were cannulated with 9 cm-long cannulae made of Intramedic Polyethylene Tubing (Clay-Adams, Parsippany, NJ, USA); a small flange (1.5 mm diameter) was formed at the tip by heating and gently pressing against a cool flat surface. The cannula was implanted in the lower right jaw with the aid of an 8-cm length of curved wire (0.25 mm diameter). One end of the wire was friction-fitted to the non-flanged end of the cannula; the other was inserted beneath the pups' tongue and it was maneuvered out the ventral surface of the jaw until the cannula was pulled through the jaw. Only the flanged end of the cannula remained inside the pups' mouth, as previously described (Kornblith and Hall, 1979). This procedure was completed in approximately 20–30 s. After cannulation, pups were transferred to a warm incubation chamber for 2 h (32–36 °C) with similarly implanted littermates. This was done to insure that all pups were tested at least 2 h after any potential analgesic effects produced by nursing and to minimize Fos expression produced by the cannulation procedure.

Midcollicular transections

Pups were anesthetized with isoflurane (2.5–3%, via spontaneous respiration) and mounted in a Cunningham neonatal rat adaptor stereotaxic instrument (Stoelting Co, Wood Dale, IL, USA). Transections were adapted from the neonatal procedure described by Kornblith and Hall (1979). Briefly, the skull was exposed and a 0.2 mm, coronally oriented opening was drilled at midline at a midcollicular-level, approximately 1.0 mm posterior to lambda. A thin, blunt knife fabricated from a stainless steel L-shaped spatula (base, 0.5 mm wide×2 mm long; shaft, 3 mm wide×10 mm long, ground to a 0.3 mm thickness) was used to make a complete midcollicular transection without damage to the superior sagittal sinus and large vessels at the base of the midbrain. This approach minimized blood loss. The craniotomy was closed and animals recovered for 40 min before subsequent testing (see below). Sham animals were treated in an identical manner but were spared the knife cut. Sham and transected animals were similar in terms of gross spontaneous motor activity, orofacial movements during intraoral infusions, and paw or tail withdrawal to nocifensive stimuli. After testing, brains were removed, fixed overnight in a 4% buffered paraformaldehyde solution, sectioned and examined to verify the extent of the transection.

Mechanical withdrawal responses

Cutaneous flexion hindpaw withdrawal responses were elicited using calibrated von Frey monofilaments as in previous studies (Ren, 1999; Anseloni et al., 2002, 2004). The filaments, ranging in bending force from 9 mg to 15 g, were applied in an ascending series. Each von Frey filament was applied to the lateral dorsum of the hindpaw for approximately 1 s and repeated five times at intervals of 2–5 s. The response frequency [(number of responses/number of stimuli)×100%] to a range of von Frey filament forces was also determined. The stimulus-response frequencies were generated from these data and were statistically compared (one-way ANOVA and ANOVA repeated measures). Non-linear regression analysis (GraphPad Prism, version 3.02, GraphPad Software, Inc., San Diego, CA, USA) was performed on stimulus-frequency curves to derive EF_{50}, the effective force (g) to produce a 50% response frequency (see Fig. 2; Anseloni et al., 2002; Guo et al., 2004). Mechanical withdrawal responses were assessed in six experimental conditions: (1) Intact animals (n=24) with intraoral cannulae, representing the pooled baseline for animals that subsequently received sham or transection surgery and intraoral infusions; (2) Post-Sham-Saline (n=6); (3) Post-Sham-Sucrose (n=6); (4) Post-Transection Baseline (n=12), representing the pooled baseline data from transection animals that subsequently received saline or sucrose infusion; (5) Post-Transection-saline (n=6); (6) Post-Transection-Sucrose (n=6). Thus, mechanical withdrawal responses were assessed twice in the pups used in sham experiments: once in the baseline condition (Intact) and then again during intraoral infusion (Post-Sham Saline/Sucrose). Nocifensive responses were assessed three times in the transection experiments: before transection (Intact), after transection (Post-Transection Baseline), and a third time during intraoral infusion (Post-Transection Saline/Sucrose). Intraoral cannulae were present in all conditions. A solution of either sucrose (7.5%) or saline (0.9%), freshly prepared in distilled water, was delivered with a motor driven syringe pump (see below). Infusions began 90 s prior to, and continued throughout, mechanical testing, which lasted approximately 10 min.

Intraoral infusions

The intraoral cannula was connected to a syringe pump for delivery of solutions. The pump drove a 1-ml syringe connected to PE-50 tubing, which in turn was friction-fitted to the intraoral cannula. Solutions were infused at body temperature (36 °C) via an infusion pump (341A Sage Instruments, Freedom, CA, USA). The infusion rate (0.06 ml/min) has previously been shown to be effective in producing analgesia with 7.5% sucrose in 10-day-old rat pups (Blass et al., 1987; Blass and Shide, 1994; Anseloni et al., 2002). For the Fos experiments below, pups received a total infusion volume of 90 ul (over 90 s), previously shown to elicit opiate receptor-dependent analgesia in 10-day old rat pups (Anseloni et al., 2002).