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MESOPONTINE TEGMENTAL ANESTHESIA AREA PROJECTS INDEPENDENTLY TO THE ROSTROMEDIAL MEDULLA AND TO THE SPINAL CORD

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Abstract—General anesthetics are presumed to act in a distributed manner throughout the CNS. However, we found that microinjection of $GABA_A$-receptor ($GABA_A$-R) active anesthetics into a restricted locus in the rat brainstem, the mesopontine tegmental anesthesia area (MPTA), rapidly induces a reversible anesthesia-like state characterized by suppressed locomotion, atonia, anti-nociception and loss of consciousness. GABA-sensitive neurons in the MPTA may therefore have powerful control over major aspects of brain and spinal function. Tracer studies have shown that the MPTA projects to the rostromedial medulla, an important reticulospinal relay for pain modulation and motor control. It also projects directly to the spinal cord. But do individual MPTA neurons project to one or to both targets? We microinjected fluorogold into the rostromedial medulla and cholera toxin b-subunit into the spinal cord, or vice versa. Neurons that were double-labeled, and hence project to both targets, were intermingled with single-labeled neurons within the MPTA, and comprised only 11.5% of the total. MPTA neurons that project directly to the spinal cord were larger, on average, than those projecting to the rostromedial medulla, differed in shape, and were much more likely to express $GABA_A$-α1Rs as assessed by receptor alpha-1 subunit immunoreactivity (51.4% vs. 18.9%). Thus, for the most part, separate and morphologically distinct populations of MPTA neurons project to the rostromedial medulla and to the spinal cord. Either or both may be involved in the modulation of nociception and the generation of atonia during the MPTA-induced anesthesia-like state.

Key words: reticulospinal, coma, MPTA, pain modulation, RVM, unconsciousness.

Many CNS functions depend on precise, often topographically organized pathways in which specific presynaptic neuronal populations project to specific postsynaptic target populations. In other systems connectivity is more diffuse, with broad collateralization to multiple postsynaptic targets. The former architecture is characteristic of systems that execute precise sensory and motor functions. The latter is characteristic of systems with a broader modulatory role, or that contribute to functions such as arousal. We have recently identified a unique, restricted zone in the upper brainstem at which microinjection of small quantities of pentobarbital and other $GABA_A$-receptor ($GABA_A$-R) active anesthetics causes an anesthesia-like state characterized by reversible loss of consciousness as assessed electrographically, as well as loss of locomotor activity and the righting reflex, atonia, and suppressed response to noxious stimuli (Devor and Zalkind, 2001; Voss et al., 2005; Sukhotinsky et al., 2007). Neurons in this zone, which we call the mesopontine tegmental anesthesia area (MPTA), are of considerable interest because they appear to have extraordinarily powerful control over major aspects of brain and spinal function. We ask here about the target-specificity of individual MPTA neurons.

Anterograde and retrograde tracer studies have shown that the MPTA has two prominent descending projections. These are projections to the rostromedial part of the medulla, notably the medullary gigantocellular (Gi) and gigantocellular pars alpha (GiA) reticular fields, as well as to the entire length of the spinal cord (Satoh, 1979; Abols and Basbaum, 1981; Edley and Graybiel, 1983; Jones and Yang, 1985; Newman, 1985; Rye et al., 1988; Matsuyama et al., 1993; Lai et al., 1999; Sukhotinsky et al., 2005, 2006). The rostromedial medulla is a well-known relay for opiate-induced antinociception (Fields et al., 2006). It is also strongly implicated in the control of movement and segmental muscle tone, as well as a variety of autonomic functions (Chase et al., 1986; Lai and Siegel, 1988; Takakusaki et al., 1989, 2001; Hajnik et al., 2000; Mason, 2001; Nason and Mason, 2004). The direct reticulospinal projection of the MPTA is primarily to pre-motor and motor areas, notably the ventral horn and intermediate gray matter. However, there is also a prominent projection to the deep layers of the dorsal horn and, more sparsely, also to dorsal horn laminae I and II (Jones and Yang, 1985; Matsuyama et al., 1993; Sukhotinsky et al., 2005, 2006). Thus, either or both of these pathways might contribute to the motor and sensory components of the anesthesia-like state generated by microinjecting pentobarbital into the MPTA.

The dual descending projections of the MPTA are known from studies that track entire cell populations. None of the studies carried out to date are able to indicate whether individual neurons contribute to one or both of

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Abbreviations: ABC, avidin–peroxidase complex; ChAT, choline acetyltransferase; CTB, cholera toxin B subunit; DAB, diaminobenzidine; DRN, dorsal raphe nucleus; FG, fluorogold; $GABA_A$-R, $GABA_A$-receptor; $GABA_A$-α1R, alpha-1 subunit of $GABA_A$-receptor; GAD, glutamic acid decarboxylase; Gi, gigantocellular reticular field; GiA, gigantocellular reticular field alpha; LDT, laterodorsal tegmental nucleus; LPT, lateral pontine tegmentum; MPTA, mesopontine tegmental anesthesia area; NRM, nucleus raphe magnus; PAG, periaqueductal gray; PBS, phosphate-buffered saline; PBS-azide, 0.02% sodium azide in phosphate-buffered saline; PBT-azide, 0.02% sodium azide in phosphate-buffered saline with 0.25% Triton X-100; PPT, pedunculopontine tegmental nucleus; Rob, nucleus raphe obscurus; Rpa, nucleus raphe pallidus; vlPAG, ventrolateral periaqueductal gray.

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these pathways. That is, do individual neurons of the MPTA project to both the rostromedial medulla and to the spinal cord, or does the MPTA contain intermingled populations of neurons that project to one or the other of these targets? We undertook to answer this question using a double retrograde tracer approach. Specifically, we microinjected contrasting retrograde tracers into the two target regions, the rostromedial medulla and the spinal cord gray matter, and asked what proportion of MPTA neurons was retrogradely labeled with both tracers. The results indicate that some neurons project to the medulla, and others to the spinal cord, but that relatively few project to both.

EXPERIMENTAL PROCEDURES

Animals and surgery

Studies were performed on adult male Wistar-derived Sabra strain rats (300–500 g) with the approval of the Institutional Animal Care Use Committee of the Hebrew University of Jerusalem and following the ethical guidelines of the International Association for the Study of Pain (Zimmermann, 1983). All efforts were made to minimize the number of animals used and their suffering. Animals were deeply anesthetized with ketamine and xylazine (85 mg/kg, and 13 mg/kg, i.p., respectively), or pentobarbital sodium (50 mg/kg, i.p.) and prepared for tracer microinjection. Following the microinjections surgical exposures were closed in layers, a topical bacteriostatic powder was applied to the incision, a prophylactic injection of penicillin was administered (50 ku/kg i.m.), and animals were returned to their original cages until the time of perfusion. Recovery was uneventful.

Tracer microinjection

Results of double labeling are based on data from seven rats in which contrasting retrograde tracers were successfully targeted to the rostromedial medulla and to the spinal gray matter, and technical aspects of the processing were satisfactory. Supplementary comparison data were available from rats in which a single retrograde tracer was injected into either the cervical spinal cord (12 rats) or into the rostromedial medulla (five rats, Table 1). For both spinal and medullary microinjections tracer was loaded into a glass micropipette with a tip of about 20–30 μm and injected using a "Nanoject variable" automatic injector (Drummond, Broomall, PA, USA).

For spinal microinjections the dorsal surface of the spinal cord was exposed by laminectomy at cervical levels (C5-7) or at both cervical, thoracic and lumbar levels, and rows of three ~30 nl microinjections of a retrograde tracer compound, separated from one another rostrocaudally by about 500 μm, were made into the spinal gray matter. For medullary microinjections the animal was mounted in a stereotaxic apparatus with the head leveled from bregma to lambda. The scalp was opened, fascia scraped, and the dura overlying the rostral medulla was exposed in a small one-sided craniectomy made using a dental burr. The injection micropipette was mounted for vertical insertion, and the dura was opened locally to facilitate penetration. Tracer injections were targeted to the rostromedial medulla, defined for present purposes as an area that comprises the nucleus raphe magnus (NRM), the nuclei raphe pallidus and obscurus (Rpa, Rob), the Gi and the GiA, according to the rat brain atlas of Paxinos and Watson (1986, and the electronic version, Paxinos and Watson, 1998). Stereotactic coordinates used were: 10.5–11.8 mm posterior to bregma, 0–1.5 mm lateral to the midline, and 9–11 mm ventral to the brain surface. An injection of 27 nl was made, the micropipette was left in place for 2–5 min to minimize tracer spread back along the pipette track, and then it was slowly withdrawn.

For the double retrograde tracer experiments either cholera toxin B subunit (CTB, 1% in saline, List Biological, Campbell, CA, USA, product #103A, lot #10327A3) was injected into the spinal gray while fluorogold (FG) (5% or 10% in 0.9% saline, Fluorochrome, Englewood, CO, USA) was injected into the rostromedial medulla (four rats), or FG was injected into the spinal cord with CTB injected into the rostromedial medulla (three rats, Table 1). Medullary and spinal injections were always on the same side. For the single retrograde tracer experiments either FG or CTB was injected into one of the two target sites (Table 1).

Tracer visualization

After 4–7 days animals were anesthetized with an overdose of chloral hydrate (450 mg/kg, i.p.) or pentobarbital (60 mg/kg, i.p.) and perfused transcardially with 0.9% saline or PBS (10 mM phosphate-buffered saline) followed by 4% formaldehyde in 0.1 M phosphate buffer both at pH 7.3 and room temperature. The brain and spinal cord were dissected out immediately and postfixed for 3–6 h in the same fixative. They were then transferred to cryoprotectant (0.02% sodium azide in phosphate-buffered saline (PBS-azide) containing 20% sucrose, 4 °C). At least 48 h later tissue was cut on a freezing microtome into six or eight consecutive series of 50 μm thick coronal sections and stored in PBS-azide at 4 °C.

To visualize retrogradely transported CTB, sections were rinsed in PBS and then incubated free floating for 2–3 days in polyclonal goat anti-CTB antibody (List Biological, product #703, lot #7032H, 1:50,000) in PBS-azide containing 0.25% Triton X-100 (PBT-azide) at room temperature. For single tracer experiments, after rinsing, sections were incubated for 60 min in biotinylated donkey anti-goat IgG (Jackson ImmunoResearch, West Grove, PA, USA; 1:1000 in PBT), rinsed, and incubated for an additional 60 min in avidin–peroxidase complex (ABC) reagent in PBS (1 μl/ml, Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA). Sections were then rinsed and placed in a PBS solution containing 0.05% DAB (diaminobenzidine), 0.01% H_2O_2 and 5% Ni $(NH_4)SO_4$ for 10–30 min with visual monitoring. For double tracer experiments sections incubated in the anti-CTB antibody were rinsed and then incubated for 2 h in Alexa Fluor 488 conjugated anti-goat IgG (Molecular Probes, Eugene, OR, USA; 1:500 in PBT). FG, the second tracer used in double tracer experiments, was recognized on the basis of its intrinsic fluorescence. In single tracer experiments in which FG was microinjected, rather than depending on intrinsic fluorescence we usually used immunolabeling to enhance sensitivity and to create a permanent record. Rinsed sections were incubated for 2–3 days in polyclonal rabbit anti-FG primary antibody (Chemicon, Temecula, CA, USA, product #AB153, lot #24110032, 1:10,000 in PBT) followed by biotinylated goat anti-rabbit secondary antibody (Jackson, 1:1000). The procedure was completed using the ABC–DAB reaction described above for CTB.

Sections containing fluorescent-labeled cells were rinsed in H_2O, mounted on gel-covered slides, air-dried and coverslipped with Immu-mount (Thermo-Shandon, Pittsburgh, PA, USA). Tissue was examined under epifluorescence microscopy with appropriate filter combinations (FG: absorption 331 nm, emission 418 nm; Alexa Fluor 488: absorption 495 nm, emission 519 nm). Sections in which cells were marked with DAB reaction product were mounted and then lightly counterstained with Cresyl Violet. They were then rinsed in H_2O, dehydrated in ascending alcohols, cleared with xylene and coverslipped using Entellan (Merck, Darmstadt, Germany). The primary antibodies used were raised against exogenous marker molecules (CTB and FG). Antibody specificity was assured by the presence of labeling only in neurons positioned to take up and transport the markers, and its absence when primary antibody was omitted, and in brains in which the tracer was not applied.