Neuronal Degeneration in Canine Narcolepsy

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Narcolepsy is a lifelong illness characterized by persistent sleepiness, hypnagogic hallucinations, and episodes of motor paralysis called cataplexy. We have tested the hypothesis that a transient neurodegenerative process is linked to symptom onset. Using the amino-cupric silver stain on brain sections from canine narcoleptics, we found elevated levels of axonal degeneration in the amygdala, basal forebrain (including the nucleus of the diagonal band, substantia innominata, and preoptic region), entopeduncular nucleus, and medial septal region. Reactive neuronal somata, an indicator of neuronal pathology, were found in the ventral amygdala. Axonal degeneration was maximal at 2–4 months of age. The number of reactive cells was maximal at 1 month of age. These degenerative changes precede or coincide with symptom onset. The forebrain degeneration that we have observed can explain the major symptoms of narcolepsy.

Key words: narcolepsy; REM sleep; amygdala; basal forebrain; canine; amino-cupric silver; degeneration; cataplexy

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MATERIALS AND METHODS

Eighteen Doberman pinscher dogs, nine narcoleptic and nine age- and breed-matched controls (four male narcoleptics and seven male controls), from six narcoleptic and five normal litters ranging from 1 to 8 months of age were used (Table 1). Control and narcoleptic dogs were reared under similar conditions and never given any pharmacological agents before killing. They were anesthetized with sodium pentobarbital (50 mg/kg) and perfused with a rinsing solution of 0.8% sucrose, 0.4% glucose, and 0.8% NaCl in 0.067 M cacodylate buffer, pH 7.3. Fixation was with 4% formaldehyde in 0.067 M cacodylate buffer containing 4% sucrose. After the brains were allowed to harden in situ for 24 hr, they were removed from the skull and placed in fixative for 7 d.

Amino-cupric protocol

Embedding and sectioning

Brains were treated with 20% glycerol and American Optical dimethylsulfoxide to prevent freeze artifacts. Two half brains (a narcoleptic and control) were embedded side by side, with their medial surfaces aligned using the anterior commissure as a landmark. The block of embedded brains was allowed to cure and then rapidly frozen by immersion in isopentane chilled to −70°C with crushed dry ice. Blocks were mounted on a freezing stage of an American Optical sliding microtome and sectioned coronally at 40 μm beginning at the olfactory bulb and proceeding to the spinal medullary junction (i.e., decussation of the pyramids). All sections cut (none were discarded) were collected sequentially into a 4 × 6 array of containers. These containers were filled with either standard 10% commercial, phosphate-buffered formaldehyde or 3.7–4% formaldehyde buffered with 4.2% sodium cacodylate, pH 7.2, for sections to be stained with the amino-cupric silver method. At the completion of sectioning, each container held a serial set of one of every 24th section (or, one section every 960 μm). Each of the large sections cut from the block was a composite section holding sections from both of the half brains.

Staining

Selection of sections for staining. A serial set of every sixth section (a 240 μm interval) was selected for staining with the amino-cupric-silver stain of de Olmos (1994) to reveal disintegrative degeneration. The free-
The time course of the degenerative process in the narcoleptic dogs paralleled the lower levels of axonal and neuronal degeneration in each brain region seen at the same stage of development in the control dogs, with both peaking at 1–3 months. In the dogs, symptom onset occurs relatively early in life, at 1–4 months of age.

**DISCUSSION**

We found that degeneration was present in canine narcoleptics at or shortly before the time of symptom onset. Degeneration was present in areas that have been implicated in response to startle and in sleep control.

No consistent evidence for neuronal or axonal degeneration has been reported in any brain region in human narcoleptics. However, a few cases of “symptomatic” narcolepsy linked to tumors or other lesions have been seen. Most patients with symptomatic narcolepsy have been reported to have diencephalic–basal forebrain–septal nucleus damage, whereas few had any brainstem pathology (Stahl et al., 1980; Erlich and Itabashi, 1986; Aldrich and Naylor, 1989; Servan et al., 1995). A recent report of brainstem lesions in narcolepsy (Plazzi et al., 1996) has been disputed (Bassetti et al., 1997; Frey and Heiserman, 1997). Gross anatomical lesions caused by neoplasms or strokes are absent in the vast majority of human cases (Aldrich, 1990).
(Mignot et al., 1993), consistent with the observed neuronal degeneration. Human narcolepsy has been seen in children as young as 3 years (Yoss and Daly, 1960; Billiard, 1985; Kotagal et al., 1990; Challamel et al., 1994) but typically starts in the second or third decade (Aldrich, 1990). Our time course data suggest two possible scenarios for a comparable degenerative process in humans. The first is that degeneration could occur at the age of disease onset with no previous abnormality. The second possibilit-

Figure 1. Examples of degenerative changes seen in canine narcolepsy. 

- **a, b**, Low-power dark-field view of amygdala of narcoleptic (a) and control (b) half brains of 4-month-old dogs. Increased numbers of labeled axons in the narcoleptic dog are visible as brightly illuminated foci in amygdala and pyriform cortex (bottom arrow) and central nucleus of the amygdala (top arrow compared with the same areas in the control half brain (arrows)). Optic tract is visible in top right of a and top left of b. 
- **c**, Higher magnification of amino-cupric silver-stained sections counterstained with neutral red Nissl showing axonal degeneration (black interrupted lines) in pyriform cortex nucleus of 3-month-old narcoleptic. Note several counterstained nonreactive neuronal soma in this and other sections (arrows). 
- **d**, is detail from area indicated in c. 
- **e**, Axonal degeneration in basalis magnocellularis nucleus of 3-month-old narcoleptic. 
- **f**, Detail (1000×) of axonal degeneration in medial nucleus of the amygdala of 2-month-old narcoleptic. 
- **g–i**, Reactive neuronal somata in the amygdala and subjacent pyriform cortex of narcoleptic dogs. g, A pair of darkly stained cells are visible in the center of a 2-month-old. 
- **h, i**, Labeled cells in the basalis parvicellularis amygdala of a 3-month-old. Scale bars: 
  - a, b, 1 mm; 
  - c, e, 50 μm; 
  - d, g–i, 25 μm; 
  - f, 10 μm.
ity is that degeneration could occur early in development in narcoleptic humans, with some subsequent degenerative or hormonal process triggering the disease at a later age.

The latter time course would resemble that of the degenerative process thought to occur in schizophrenia. Schizophrenia, like narcolepsy, is correlated with degeneration that includes portions of the amygdala and other frontotemporal regions (Bogerts, 1993; Marsh et al., 1994; Nasrallah et al., 1994). The best evidence is that the damage in schizophrenics occurs prenatally or early in development (Bogerts, 1993), as we find in canine narcolepsy. Like narcolepsy, symptoms of schizophrenia are usually not present in early childhood. Symptom onset in schizophrenics is typically in the second or third decade and, as in narcolepsy, damage does not appear to be progressive (Marsh et al., 1994). Most narcoleptics have hypnagogic hallucinations, a symptom with some resemblance to the hallucinatory mentation of certain schizophrenics. Several cases of schizophrenia coexisting with or misdiagnosed as narcolepsy have been reported (Cadieux et al., 1985; Douglass et al., 1991).

The amygdala is one of the forebrain areas most strongly
Figure 3. Anatomical distribution of degenerating axons in a pair of half brains from 6-month-old narcoleptic and control dogs processed together.
Figure 4. Time course of levels of axonal degeneration with age in narcoleptic and control dogs. All data are from the same group of 18 canines. There was one narcoleptic and one control at each data point except for two of each at 2 months of age.
activated in REM sleep (Maquet et al., 1996; Nofsginer et al., 1997). Amygdala stimulation in normal cats potently increases REM sleep duration (Calvo et al., 1996). The amygdala is also known to be involved in the elaboration of emotional responses and has a powerful role in the modulation of startle (Campeau and Davis, 1995). There are major projections from the amygdala to the dorsolateral pontine cholinergic and noradrenergic cell regions involved in the generation of REM sleep phenomena (Wallace et al., 1992). We hypothesize that the loss of neurons within the amygdala, basal forebrain, and septal region disinhibits amygdala cells projecting to the brainstem. These disinhibited cells are activated during sudden, strong emotions. This triggers the brainstem motor inhibitory system and inactivates the locus coeruleus (Wu et al., 1998), resulting in cataplexy. It has been shown that activation of the amygdala produces EKG acceleration and apnea (Frysinger et al., 1984), changes that also occur at the onset of cataplexy (Siegel et al., 1989).

The entopeduncular nucleus, like the amygdala, is important in the elaboration of emotional responses and has a particularly important role in the recognition of rewarding events (Hammer et al., 1993; Breiter et al., 1997). Pleasurable stimuli, including food ingestion, the most reliable trigger of canine cataplexy, activate the entopeduncular nucleus (Lidsky, 1975; Schneider, 1987). As in the amygdala, degenerative changes that alter circuitry or disinhibit cells could be responsible for an abnormal output from this region to the amygdala and brainstem regions (Schneider, 1987).

The septal nucleus is known to have important arousal and startle-related functions. Electolytic lesions of the septum produce a dramatic exaggeration of the startle response (McCleary, 1961). Cholinergic and GABAergic neurons localized to the medial septal region project to limbic structures and produce the theta rhythm in the hippocampus (Vertes and Kocsis, 1997), a rhythm that is prominent in both REM sleep and cataplexy (Wu et al., 1998).

The amygdala, diagonal band of Broca, and magnocellular preoptic region are the major components of the basal forebrain hypnogenic region. Sleep-active neurons, hypothesized to be involved in sleep induction, are localized to this area (Szymusiak and McGinty, 1986b). Stimulation of the ventral amygdala produces EEG synchrony (Kreindler and Steriade, 1964). Stimulation of the preoptic region also induces sleep (Sterman and Clemente, 1962). Lesions of this area produce the most profound insomnia seen after any brain lesion (Szymusiak and McGinty, 1986a). Narcoleptic canines have elevated levels of dopaminergic and noradrenergic receptors in the amygdala, brainstem, and basal forebrain (Melford et al., 1983; Kilduff et al., 1986). Similar changes are present in human narcoleptics (Aldrich et al., 1992, 1993, 1994). Cholinergic stimulation of the basal forebrain triggers cataplexy in narcoleptic, but not in control canines (Nishino et al., 1995). Disinhibition of the basal forebrain region by loss of local interneurons could produce the major non-REM sleep-related symptoms of narcolepsy, disruption of nighttime sleep and excessive daytime sleepiness (Aldrich, 1991), as well as the reported changes in receptor levels. Thus, the degeneration we see in amygdala, basal forebrain, and septum are consistent with the EEG, motor, and sleepiness symptoms of narcolepsy.

Human narcolepsy is correlated with the presence of the human leukocyte antigen (HLA) DQB1*0602 genotype (Matsuki et al., 1992). The association of narcolepsy with the major histocompatibility complex marker, HLA-DR2 and DQB1*0602, is one of the highest disease-HLA linkages known (Behar et al., 1995). Most HLA-linked disorders have been shown to be autoimmune in nature (Sinha et al., 1990). Canine narcolepsy is linked to the presence of a marker for an Ig switch-like sequence (Mignot et al., 1991) and enhanced microglial expression (Tafti et al., 1996) at 1–3 months of age. These findings all suggest that immune processes, perhaps related to axonal pruning or cell necrosis, may be linked to narcolepsy onset. Consistent evidence for immune abnormalities in human and canine narcolepsy have not been found, indicating that narcolepsy probably does not involve long-term generalized autoimmune activation (Fredrikson et al., 1990; Mignot et al., 1995). However, autoimmune processes linked to a localized, time-limited degenerative process, preceding symptom onset would be missed by the techniques used to look for autoimmune processes in previous studies. The degenerative changes we have observed could form the link between autoimmune activation and the abnormalities of motor and sleep function that characterize narcolepsy.
Figure 6. Distribution of reactive neuronal somata in 3- and 4-month-old narcoleptic and normal dogs. Number of reactive somata is indicated below each series.
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