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The consequences of loss followed by recovery of noradrenergic nerve function on muscarinic receptors in the chick expansor secundariorum muscle

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1 The effects of chemical sympathectomy with 6-hydroxydopamine on the response of the expansor secundariorum muscle to noradrenergic nerve stimulation, noradrenaline and acetylcholine have been investigated.

2 Expansor muscles from 60 day old chicks were sensitive to noradrenergic nerve stimulation and exogenous noradrenaline but virtually unresponsive to acetylcholine.

3 Chemical sympathectomy with 6-hydroxydopamine caused loss of function of noradrenergic nerves of the expansor muscle, induced supersensitivity to exogenous noradrenaline and gradually increased the response of the expansor muscle to acetylcholine.

4 As the patency of noradrenergic nerves reappeared there was a decline in the extent of supersensitivity to noradrenaline and the response to acetylcholine gradually declined.

5 The time courses of these changes differed, indicating that the mechanisms responsible for changes in response to noradrenaline and acetycholine are different.

Introduction

The expansor secundariorum muscle (ESM), a smooth muscle in the wing of birds, is innervated by postganglionic noradrenergic nerves only (Buckley & Wheater, 1968; Bennett & Malmfors, 1970; Lot & Bennett, 1982b). However, the ESM also possesses muscarinic receptors in chicks younger than 10 days post-hatching; these receptors disappear by day 40 post-hatching (Kuromi & Hasegawa, 1975; Bennett *et al.*, 1982).

In chicks older than 40 days, surgical denervation or sympathectomy with guanethidine gradually increases the sensitivity of the ESM to acetylcholine (ACh) (Kuromi & Hagihara, 1976; Bennett *et al.*, 1982). The supersensitivity following surgical denervation is associated with hypertrophy of the ESM (Campbell *et al.*, 1977; Lot & Bennett, 1982a) making this procedure unsuitable in the present study.

Administration of 6-hydroxydopamine (6-OHDA) has been shown to cause structural degeneration of noradrenergic nerves of the ESM (Bennett *et al.*, 1970), although no functional studies were undertaken. Since 6-OHDA causes degeneration of noradrenergic nerves which subsequently recover (Bennett *et al.*, 1973; Bennett & Malmfors, 1974) this drug would appear to be suitable for studying the influence, on muscarinic receptors, of recovery of the noradrenergic innervation of the ESM.

In the present work, the time course of changes in the sensitivity of the ESM to noradrenergic nerve stimulation, noradrenaline (NA) and ACh following administration of 6-OHDA have been investigated by use of pharmacological techniques.

Methods

Male chicks (Light Sussex and Rhode Island Red cross) aged 60 days were used in the present study.

Administration of 6-hydroxydopamine

Sixty-day old chicks were injected intravenously with 6-OHDA (100 mg kg^{-1} dissolved at a concentration of 100 mg ml^{-1} in sterile saline solution containing ascorbic acid, 0.2 mg ml^{-1}). Control animals were injected with an equivalent volume of the vehicle. Injections were given through the brachial vein. Subsequently, the animals were killed at different times by an

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overdose of ether, the ESM removed and set up in organ baths as described by Bennett *et al.* (1982).

The organ bath contained 20 ml of freshly prepared physiological solution (composition (mM): NaCl 118, KCl 4.7, $MgSO_4$ $7H_2O$ 2.4, NaH_2PO_4 1, $NaHCO_3$ 30, glucose 11.1, $CaCl_2$ 2.5) gassed with a mixture of 5% CO_2 and 95% O_2. The bath was maintained at 37°C and the tissues were set up under a resting tension of 1 g and left for 30 min to equilibrate.

Parallel platinum wire electrodes were arranged either side of the tissue and connected to a constant voltage square wave stimulator (S.R.I.). Electrical stimuli were delivered as pulses of 140 V and 0.2 ms duration for 10 s every 4 min. The frequency of stimulation was varied as desired. These parameters have been shown to stimulate selectively the noradrenergic nerves of the ESM (Lot & Bennett, 1982b).

Drug solutions were freshly prepared in concentrations such that the addition of 0.2 ml gave the final desired bath concentration (M). Ascorbic acid (1×10^{-4}M) was added to dilute solutions of NA. The drug contact times were 30 s for NA and 45 s for ACh with an interval between successive doses of at least 5 min. Concentration-response curves were established by graded increases in the concentrations of NA or ACh; frequency-response curves by graded increases in frequency of stimulation.

Drugs

Drugs used were acetycholine bromide (British Drug Houses), atropine sulphate (Sigma), (−)-noradrenaline bitartrate (Sigma) and 6-hydroxydopamine (Sigma).

Statistical analysis

Regression lines with confidence limits were calculated for the linear portions of log concentration-response curves. The significance of difference in slope was used as a measure of parallelism of the two lines.

Log concentration or frequency limits at 50% of the maximum response were used in the analysis of the significance of concentration or frequency differences as described by Birmingham *et al.* (1970).

Maximum responses were compared by means of Student's unpaired *t* test.

Results

Field stimulation

6-OHDA abolished the response of the ESM to field stimulation 1 day after administration (Figure 1a).

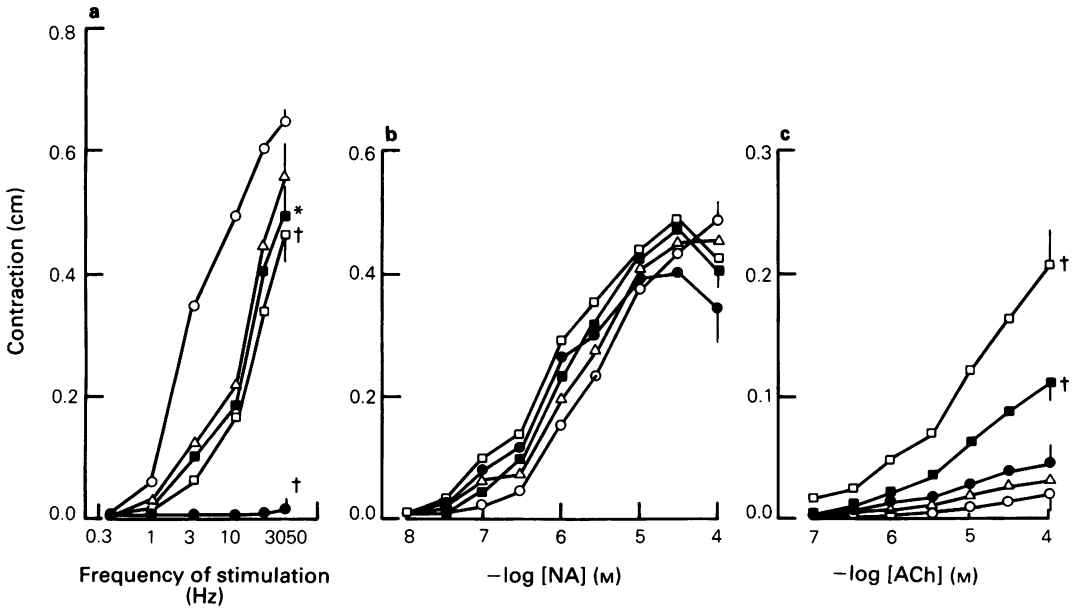

Figure 1 Mean response of expansor secundariourum muscles (ESMs) from saline-treated (control) chicks (○, $n = 24$) and from chicks 1 day (●, $n = 6$), 7 days (□, $n = 6$), 14 days (■, $n = 6$) or 28 days (△, $n = 6$) after treatment with 6-hydroxydopamine, to (a) nerve stimulation, (b) noradrenaline (NA) and (c) acetylcholine (ACh). Note that the ordinate scale in (c) is an expanded scale. Vertical lines show s.e.mean. †$P < 0.001$, *$0.01 > P > 0.001$, Student's unpaired *t* test (compared to the control).

This was followed by recovery of noradrenergic nerve function with time. Thus, the ESM responded to nerve stimulation 7 days after 6-OHDA although the curve obtained lay to the right of ($P < 0.001$), and had a depressed maximum response ($P < 0.001$) compared to the control (Figure 1a). This indicates a reappearance of noradrenergic nerve function in the ESM 7 days after treatment. ESMs taken 14 days after 6-OHDA responded to nerve stimulation although the log frequency-response curve lay to the right of ($P < 0.001$), with a depressed maximum response ($0.01 > P > 0.001$) compared to the control (Figure 1a). The position of the log frequency-response curve 28 days after 6-OHDA lay to the right of ($P < 0.001$), but the maximum recorded response did not differ significantly from that of the control (Figure 1a).

Noradrenaline

The log concentration-response curve for NA 1 day after 6-OHDA was parallel and lay to the left ($P < 0.001$) of the control (Table 1). This supersensitivity to NA was not accompanied by a significant change in the maximum response to NA (Figure 1b).

Although there was evidence that noradrenergic nerve function had reappeared in the ESM 7 days after 6-OHDA, the muscle was still supersensitive to NA (Figure 1b; Table 1). This supersensitivity to NA was comparable to that seen 1 day after 6-OHDA (Table 1) and was not accompanied by a significant change in the maximum response (Figure 1b).

The log concentration-response curves for NA both 14 days and 28 days after 6-OHDA did not differ significantly from the control (Figure 1b; Table 1).

Acetylcholine

The control ESM was virtually unresponsive to ACh. The response of the ESM to ACh 1 day after 6-OHDA did not differ significantly from the control (Figure 1c). The ESM was more responsive to all doses of ACh tested both 7 and 14 days after 6-OHDA treatment (Figure 1c). Figure 1c also shows that the largest response of the ESM to ACh was observed 7 days after 6-OHDA treatment. Thus, the maximum response of the ESM to ACh 14 days after 6-OHDA was only 0.12 ± 0.01 cm while that seen 7 days after 6-OHDA was 0.21 ± 0.03 cm ($n = 6$); this difference was significant ($0.02 > P > 0.01$).

The mean response to ACh of the ESMs taken 28 days after 6-OHDA did not differ from the control (Figure 1c) although the muscles from 2 out of 6 chicks still responded to ACh.

All responses of the ESM to ACh were completely abolished by 1×10^{-6}M atropine ($n = 6$).

Table 1 Noradrenaline sensitivities of the expansor secundariorum muscle following 6-hydroxydopamine treatment

Time after injection (days)	*Noradrenaline* ED_{50}
Control	$3.1 \pm 1.3 \times 10^{-6}$M
1	$7.4 \pm 1.5 \times 10^{-7}$M†
7	$8.3 \pm 1.3 \times 10^{-7}$M†
14	$1.3 \pm 1.3 \times 10^{-6}$M
28	$1.7 \pm 1.2 \times 10^{-6}$M

Values shown are mean ± s.e.mean of 6 chicks in each group. †$P < 0.001$, Student's unpaired t test (compared to the control).

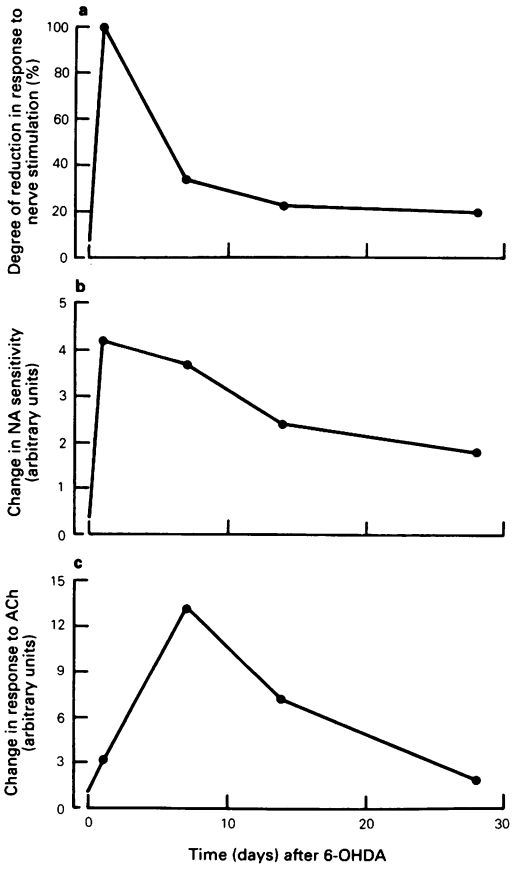

Figure 2 Time course for changes in response to nerve stimulation (a), noradrenaline (NA,b) or acetylcholine (ACh,c) of expansor secundariourum muscles (ESMs) taken at different times after 6-hydroxydopamine (6-OHDA) treatment.

Time course of the effects of 6-OHDA

The time course of the effects of 6-OHDA on the ESM calculated from mean values of the measured variables are represented in Figure 2. Note that the acute loss of noradrenergic nerves, 1 day after 6-OHDA resulted in the maximum recorded supersensitivity to exogenous NA. Subsequently the extent of these effects declined with time. However, the time course of changes in response of the ESM to ACh were different since the sensitivity to ACh returned gradually, reaching a maximum 7 days after 6-OHDA treatment. This responsiveness then gradually declined.

Discussion

In the present study it was shown that noradrenergic nerves became non-functional 1 day after treatment with 6-OHDA. This is consistent with previous observations on the time course for the degeneration of the noradrenergic nerves in chicks (Bennett *et al.*, 1970; 1973). Although regeneration of the noradrenergic innervation of the ESM has not previously been demonstrated, results from the present study indicate that the nerves are functional 7 days after 6-OHDA. This was followed by a gradual improvement in their function.

Noradrenaline (NA) supersensitivity following 6-OHDA treatment has previously been found in atrial tissue from chicks (Bennett & Malmfors, 1974). In that study, the supersensitivity observed between 1–7 days after 6-OHDA was attributed to the loss of uptake sites. NA supersensitivity following 6-OHDA, which was attributed to the loss of uptake sites has also been described for rats (Nadeau *et al.*, 1971). The degree of supersensitivity is similar to that seen following inhibition of neuronal uptake with cocaine or following short-term surgical denervation (Trendelenburg, 1963; 1966; Lot & Bennett, 1982a). The supersensitivity to NA seen during the present study occured 1–7 days after 6-OHDA and can also be attributed to the acute loss of noradrenergic nerves, hence uptake sites.

It has been shown, using chick atria, that the time course of functional recovery from the side effects of 6-OHDA appears to reflect the rate of regeneration of destroyed noradrenergic nerves (Bennett & Malmfors, 1974). However, in the present study it was shown that the ESM was still supersensitive to NA 7 days after 6-OHDA, although there was evidence for reappearance of nerve function. This could indicate that some nerves had recovered by day 7 and could release NA, but that there were, possibly, too few to take up exogenous NA effectively. By 14 days after 6-OHDA, the ESM was no longer supersensitive to NA, most likely because of an increase in the number of functional nerves and uptake sites. These results are consistent with previous findings which show that chick embryo hearts possess the capacity to concentrate exogenous tritiated NA and that this capacity increases with age (Ignarro & Shideman, 1968b), possibly due to proliferation of nerves (Ignarro & Shideman, 1968a). Another factor that could contribute to the gradual functional recovery of the nerves is the efficiency of the uptake mechanism. This is so since the extent of degeneration of noradrenergic nerves has been demonstrated to be greater in older than in younger chicks following equivalent doses of 6-OHDA, due to the greater uptake efficiency of the older nerves (Bennett *et al.*, 1973). It is therefore possible that the regenerating nerves of chicks have a less efficient uptake mechanism which improves with time.

ESMs from 60 day old chicks were virtually unresponsive to acetylcholine (ACh). The loss of function of noradrenergic nerves of the ESM following 6-OHDA restored its response to ACh. Following recovery of noradrenergic nerve function there was a progressive decrease in the response of the muscle to ACh. All responses to ACh were mediated by stimulation of muscarinic receptors since they were always abolished by atropine, a known muscarinic receptor antagonist. These findings strengthen the suggestion that the noradrenergic innervation of the ESM influences the number of muscarinic receptors expressed in the tissue (Bennett *et al.*, 1982).

Comparisons of the time courses of changes in sensitivity of the ESM to nerve stimulation, NA or ACh (Figure 2) showed that 1 day after treatment with 6-OHDA the nerve-mediated contraction of the ESM was abolished; the supersensitivity to NA had reached its peak due to the acute loss of uptake sites but there was no appreciable change in response to ACh. This suggests that changes in ACh sensitivity are unlikely to be related to the acute loss of noradrenergic nerve function. With evidence of reappearance of noradrenergic nerves in the ESM 7 days after 6-OHDA, there was a slight decrease in NA supersensitivity but the response to ACh had risen to its highest value. These findings indicate that the sensitivity of the ESM to ACh is not immediately suppressed by the restitution of noradrenergic nerves. The observations show that the mechanisms responsible for changes in sensitivity of the ESM to NA and ACh are different. This is consistent with previous suggestions that NA supersensitivity is due to the loss of uptake sites (Trendelenburg, 1963; 1966; Bennett & Malmfors, 1974; Lot & Bennett, 1982a) while the increased response to ACh is due to an increase in the number of postsynaptic muscarinic receptors (Bennett *et al.*, 1982).

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References

BENNETT, T., BURNSTOCK, G., COBB, J.L.S. & MALMFORS, T. (1970). An ultrastructural and histochemical study of the short-term effects of 6-hydroxydopamine on adrenergic nerves in the domestic fowl. *Br. J. Pharmac.*, **38,** 802–809.

BENNETT, T., LOT, T.Y. & STRANGE, P.G. (1982). The effects of noradrenergic denervation on muscarinic receptors of smooth muscle. *Br. J. Pharmac.*, **76,** 177–183.

BENNETT, T. & MALMFORS, T. (1970). The adrenergic nervous system of the domestic fowl (*Gallus domesticus L.*). *Z. Zellforsch.*, **106,** 22–50.

BENNETT, T. & MALMFORS, T. (1974). Regeneration of the noradrenergic innervation of the cardiovascular system of the chick following treatment with 6-hydroxydopamine. *J. Physiol.*, **242,** 517–532.

BENNETT, T., MALMFORS, T. & COBB, J.L.S. (1973). Fluorescence histochemical study of the degeneration and regeneration of the noradrenergic nerves in the chick following treatment with 6-hydroxydopamine. *Z. Zellforsch.*, **142,** 103–130.

BIRMINGHAM, A.T., PATERSON, G. & WOJCICKI, J. (1970). A comparison of the sensitivities of innervated and denervated rat vasa deferentia to agonist drugs. *Br. J. Pharmac.*, **39,** 748–754.

BUCKLEY, G.A. & WHEATER, L.D. (1968). The isolated expansor secundariorum – a smooth muscle preparation from the wing of the domestic fowl. *J. Pharm. Pharmac.*, **20** (suppl.), 114S–121S.

CAMPBELL, G.R., GIBBINS, I., ALLAN, I. & GANNON, B. (1977). Effects of long-term denervation on smooth muscle of the chicken expansor secundariorum. *Cell Tiss. Res.*, **176,** 143–156.

IGNARRO, L.J. & SHIDEMAN, F.E. (1968a). Norepinephrine and epinephrine in the embryo and embryonic heart of the chick: uptake and subcellular distribution. *J. Pharmac. exp. Ther.*, **159,** 49–58.

IGNARRO, L.J. & SHIDEMAN, F.E. (1968b). The requirement of sympathetic innervation for the active transport of norepinephrine by the heart. *J. Pharmac. exp Ther.*, **159,** 59–65.

KUROMI, H. & HAGIHARA, Y. (1976). Influence of sympathetic nerves on development of responsiveness of the chick smooth muscle to drugs. *Eur. J. Pharmac.*, **36,** 55–59.

KUROMI, H. & HASEGAWA, S. (1975). Changes in acetylcholine and noradrenaline sensitivity of chick smooth muscle wholly innervated by sympathetic nerve during development. *Eur. J. Pharmac.*, **33,** 41–45.

LOT, T.Y. & BENNETT, T. (1982a). Comparison of the effects of chronic chloroquine treatment and denervation on noradrenergic mechanisms. *Med. Biol.*, **60,** 25–33.

LOT, T.Y. & BENNETT, T. (1982b). Comparison of the effects of chloroquine, quinacrine and quinidine on autonomic neuroeffector mechanisms. *Med. Biol.*, **60,** 307–317.

NADEAU, R.A., de CHAMPLAIN, J. & TREMBLAY, G.M. (1971). Supersensitivity of the isolated rat heart after chemical sympathectomy with 6-hydroxydopamine. *Can. J. Physiol. Pharmac.*, **49,** 36–44.

TRENDELENBURG, U. (1963). Supersensitivity and subsensitivity to sympathomimetic amines. *Pharmac. Rev.*, **15,** 225–276.

TRENDELENBURG, U. (1966). Mechanisms of supersensitivity and subsensitivity to sympathomimetic amines. *Pharmac. Rev.*, **18,** 629–640.

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